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Low-Pressure Chromatographic Flow Systems with Amperometric Detection and DLLME-Based Methodologies for Coffee Analysis

Alexandra Maria Rangel Barbosa Poças da Silva

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Doctoral Program in Sustainable Chemistry
Department of Chemistry and Biochemistry
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Alexandra Maria Rangel Barbosa Poças da Silva

Thesis carried out as part of the Doctoral Program in Sustainable Chemistry

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To those whose light guided me, even beyond their time,

I am a reflection of all my stars

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Publications List

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The results presented in this thesis were published in international scientific journals. One of the works developed during a stay in the University of Santiago de Compostela, namely “Green solvents in dispersive liquid-liquid microextraction for the determination of carbonyl compounds in coffee extracts”, was awarded in the program **IACOBUS – Publicações Científicas (Papers)**, edition 2025/2026.

Publications in International Scientific Journals Related to This Thesis

1. Silva; Alexandra R.; Almeida, Paulo J.; Santos, João R. Multiple pulse amperometry in low pressure chromatography for parallel determination of oxidizable and reducible compounds. Analysis of a green coffee extract as a case study. *Talanta* 2023, 266:125016. https://doi.org/10.1016/j.talanta.2023.125016

2. Silva; Alexandra R.; Almeida, Paulo J.; Santos, João R. Fan assisted extraction and low pressure chromatographic system with a phenyl monolithic column for amperometric determination of volatile α-dicarbonyl compounds in coffee brews. *Microchemical Journal* 2024, 207:222689. https://doi.org/10.1016/j.microc.2024.111689

3. Custodio-Mendoza, J.A.; Silva, Alexandra R.; Kurek, M.A.; Almeida, P.J.; Santos, J.R.; Rodrigues, J.A.; Carro, A.M. Assessment of Lipid Peroxidation Products in Adult Formulas: GC-MS Determination of Carbonyl and Volatile Compounds Under Different Storage Conditions. *Foods* 2024, 13:3752. https://doi.org/10.3390/foods13233752

4. Silva, Alexandra R.; Custodio-Mendoza, J.A.; Santos, J.R; Almeida, P.J.; Rodrigues, J.A.; Carro, A.M. Green solvents in dispersive liquid-liquid microextraction for the

determination of carbonyl compounds in coffee extracts. *Journal of Chromatography* A 2025, 1745:465743. https://doi.org/10.1016/j.chroma.2025.465743

5. Silva, Alexandra R.; Santos, J. R. Feasibility of low-pressure chromatographic flow injection systems. Experimental concerns and analytical applications. *Talanta* 2025, 300:129214. https://doi.org/10.1016/j.talanta.2025.129214

Publications in International Scientific Journals Not Included in This Thesis

1. Custodio-Mendoza, J.A., Rangel Silva, A., Pokorski, P., Aktaş, H., Kurek, M.A. Green sample preparation for anthocyanin extraction from purple corn analytical evaluation of pressurized liquid and ultrasound-assisted extraction using sustainable solvents. *Analytical and Bioanalytical Chemistry* 2025, 417 (18):4025-4037. https://doi.org/10.1007/s00216-025-05951-8

Resumo

A crescente procura por métodos rápidos, fiáveis e ecológicos tem levado ao desenvolvimento de abordagens analíticas alinhadas com os princípios de química verde. Esta premissa, aliada ao objetivo de desenvolver metodologias de baixo custo, posicionou os sistemas cromatográficos de fluxo de baixa pressão como alternativa promissora às técnicas cromatográficas tradicionais de alta pressão devido à simplicidade instrumental e ao reduzido consumo de solventes e energia. Neste contexto, a presente investigação foca-se no desenvolvimento, avaliação e aplicação de sistemas cromatográficos de fluxo de baixa pressão com deteção eletroquímica para a determinação de analitos em amostras de café. Simultaneamente, foram também desenvolvidas estratégias sustentáveis para preparação de amostras baseadas na microextração líquido-líquido dispersiva assistida por ultrassons para a determinação de diversos analitos em matrizes alimentares.

Os fundamentos conceptuais desta investigação alicerçam-se numa extensa revisão analítica sobre cromatografia por injeção em fluxo de baixa pressão, na qual é discutida a evolução dos sistemas analíticos baseados em injeção de fluxo com colunas cromatográficas. Apresenta-se uma síntese das estratégias de propulsão da fase móvel, modos de injeção de amostras e sistemas de deteção, juntamente com os componentes auxiliares utilizados para otimizar o desempenho analítico do sistema. Esta revisão resume ainda as aplicações reportadas de acordo com os mecanismos de separação e os parâmetros de desempenho analítico, identificando limitações atuais e perspetivas futuras para promover o desenvolvimento geral de abordagens cromatográficas de baixa pressão.

No âmbito desta tese, realizaram-se dois estudos experimentais através da implementação de sistemas cromatográficos de baixa pressão com colunas monolíticas de comprimento curto, acoplados à deteção amperométrica, para a determinação de diferentes analitos em amostras de café. Primeiramente, utilizou-se uma coluna monolítica C_{18} com 1 cm de comprimento para a separação cromatográfica de ácidos clorogénicos, cafeína e trigonelina. Com recurso à deteção amperométrica de múltiplos pulsos, foi possível a deteção paralela de analitos passíveis de serem oxidados (nomeadamente os ácidos clorogénicos e a cafeína) e de analitos passiveis de serem reduzidos (trigonelina). Num segundo estudo, selecionou-se uma coluna monolítica de fenilo com 0,5 cm de comprimento para a separação cromatográfica de compostos α-dicarbonílicos, extraídos de infusões de café utilizando a estratégia de extração por

convecção gasosa assistida por ventoinha (*fan-assisted extraction*). A metodologia desenvolvida permitiu a quantificação de diacetilo e 2,3-pentanodiona em infusões de café. Os resultados de ambos os estudos destacaram a compatibilidade dos sistemas cromatográficos de baixa pressão com a deteção eletroquímica, bem como a rentabilidade e o baixo impacto ambiental destes sistemas analíticos.

Complementarmente, exploraram-se estratégias sustentáveis de preparação de amostra, baseadas na microextração líquido-líquido dispersiva assistida por ultrassons, previamente à análise por cromatografia gasosa acoplada a espetrometria de massa. Estas abordagens foram aplicadas na determinação de compostos orgânicos voláteis em fórmulas nutricionais para adultos, armazenadas sob várias condições, permitindo avaliar os produtos de peroxidação lipídica. Adicionalmente, a substituição de solventes de extração convencionais por alternativas mais ecológicas e menos tóxicas permitiu a extração e concentração de nove compostos carbonílicos voláteis em amostras de café. Através da métrica AGREEprep, corroborou-se a sustentabilidade das metodologias desenvolvidas, tendo-se observado uma redução no impacto ambiental sem comprometer o desempenho analítico.

Em suma, esta tese ilustra o potencial dos sistemas cromatográficos de baixa pressão com deteção eletroquímica na implementação de metodologias analíticas sustentáveis como alternativas aos métodos convencionais de HPLC para matrizes alimentares complexas. Adicionalmente, esta tese contribui para o desenvolvimento de estratégias de preparação de amostra sustentáveis, visando um baixo impacto ambiental enquanto melhora o desempenho analítico.

Palavras-chave: Cromatografia de baixa pressão, Sistemas analíticos de fluxo, Deteção eletroquímica, Química analítica verde, Microextração assistida por ultrassons, Compostos carbonílicos, Cafeína, Ácidos clorogénicos, Trigonelina, Café, Análise de alimentos

Abstract

The demand for fast, reliable and environmentally friendly methods has led to an increasing development of analytical approaches aligned with the principles of green chemistry. This need, coupled with the goal to develop low-cost methodologies, has turned low-pressure chromatographic flow systems into an alternative to the traditional high-pressure chromatographic techniques, due to their reduced instrumentation requirements, as well as low solvent and energy consumption. In this context, the present research is focused on the development, evaluation, and application of low-pressure chromatographic flow systems with electrochemical detection for the determination of analytes in coffee samples. Concurrently, green sample preparation strategies based on ultrasound-assisted dispersive liquid-liquid microextraction were also developed, for the determination of several analytes in food matrices.

The conceptual aspects of this research come from an extensive analytical review on low-pressure flow injection chromatography, where the evolution of flow injection based analytical systems with chromatographic columns is discussed. A summary of the mobile phase propulsion strategies, sample injection modes, and detection systems is provided, along with the employed auxiliary components to optimize the system's analytical performance. This review further summarizes the reported applications according to separation mechanisms, and analytical figures of merit, identifying current limitations and future perspectives to promote the general development of low-pressure chromatographic approaches.

In this thesis, two experimental studies were performed by implementing low-pressure chromatographic systems with short length monolithic columns, coupled to amperometric detection, for the determination of different analytes in coffee samples. Firstly, a 1 cm-length C_{18} monolithic column was used for the chromatographic separation of chlorogenic acids, caffeine, and trigonelline. By using multiple amperometric detection, the parallel detection of oxidizable (such as chlorogenic acids and caffeine) and reducible analytes (trigonelline) was possible. In a second case-study, a 0.5 cm-length phenyl monolithic column was selected for the chromatographic separation of α-dicarbonyl compounds, extracted from coffee brews using the fan-assisted extraction strategy. The developed methodology allowed the quantification of diacetyl and 2,3-pentanedione in coffee brews. Results from both studies highlighted the compatibility of low-pressure chromatographic systems with electrochemical detection,

as well as the cost-effectiveness and low environmental impact of these analytical systems.

An additional development in this thesis involved the study of sustainable sample preparation strategies, based on ultrasound-assisted dispersive liquid-liquid microextraction prior to gas chromatography-mass spectrometry analysis. Specifically, these approaches were employed in the determination of volatile organic compounds in adult nutritional formulas, stored under various conditions, allowing for the assessment of lipid peroxidation products. Furthermore, by replacing conventional extraction solvents with greener and less toxic alternatives, it was possible to extract and concentrate nine volatile carbonyl compounds from coffee samples. Using the AGREEprep metric, the greenness of the developed methodologies was assessed, and a reduction in environmental impact was observed without compromising analytical performance.

Thus, this thesis illustrates the potential of low-pressure chromatographic systems with electrochemical detection for implementing sustainable analytical methodologies as alternatives to conventional HPLC methods for complex food matrices. Furthermore, this thesis contributes to the development of sustainable and environmentally friendly sample preparation strategies, aiming for a low environmental impact while improving analytical performance.

Keywords: Low-pressure chromatography, Flow-based analytical systems, Electrochemical detection, Green analytical chemistry, Ultrasound assisted microextraction, Carbonyl compounds, Caffeine, Chlorogenic acids, Trigonelline, Coffee, Food analysis.

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List of Abbreviations

α-DC	α-Dicarbonyl Compounds
ACE	Acetaldehyde
ACE-d4	Tetradeuteroacetaldehyde
ACN	Acetonitrile
ACRL	Acrolein
BW	Body Weight
CGA	Chlorogenic Acid
CQA	Caffeoylquinic Acid
CV	Coefficient of Variation
DA	Diacetyl
DAD	Diode-Array Detector
DES	Deep Eutectic Solvents
DLLME	Dispersive Liquid-Liquid Microextraction
DNPH	2,4-Dinitrophenylhydrazine
EtOH	Ethanol
FAE	Fan-Assisted Extraction
FCHO	Formaldehyde
FICS	Flow Injection Chromatographic System
GC	Gas Chromatography
GCE	Glassy Carbon Electrode
GDME	Gas Diffusion Microextraction
GO	Glyoxal
HETP	Height Equivalent to a Theorical Plate
HF	High Flow
HPLC	High-Performance Liquid Chromatography
HS	Headspace
IPR	Ion Pair Reagent
IS	Internal Standard

ISA	Ionic Strength Adjustment
LC	Liquid Chromatography
LC	Low Flow
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MDA	Malondialdehyde
MeOH	Methanol
MGO	Methylglyoxal
MPA	Multiple Pulse Amperometry
MS	Mass Spectrometry
NPD	Nitrogen-Phosphorus Detector
OPDA	Ortho-Phenylenediamine
PFHpA	Perfluoroheptanoic Acid
PFPH	Pentafluorophenyl Hydrazine
PhCHO	Benzaldehyde
PTFE	Polytetrafluoroethylene
Rs	Resolution
RSD	Relative Standard Deviation
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
TBA	2-Thiobarbituric Acid
UA-DLLME	Ultrasound Assisted Dispersive Liquid-Liquid Microextraction
UV-Vis	Ultraviolet-Visible
VOC	Volatile Organic Compound

Objectives

The main aim of this PhD thesis was to implement and evaluate sustainable analytical strategies for the determination of relevant compounds in food matrices. To this end, focus is given to the implementation and assessment of low pressure chromatographic (LPC) systems and to the development of greener extraction methodologies as alternatives to conventional solvent-intensive consumption approaches.

Two main lines of research were therefore defined:

1. Development and evaluation of LPC systems

This line of investigation focused on the design, optimization, and analytical validation of LPC systems with electrochemical detection as sustainable alternatives to conventional high-pressure chromatographic techniques.

The specific goals included:

- Critical review of the progression and development of LPC systems, including the configuration of analytical systems, assessment of chromatographic parameters, detection approaches, and analytical applications, identifying current limitations and future perspectives.
- Development of a LPC system for the parallel determination of oxidizable and reducible compounds (trigonelline, caffeine, and chlorogenic acids) in coffee samples, using multiple pulse amperometry.
- Development of a LPC system with multiple amperometric detection for the determination of volatile α-dicarbonyl compounds previously extracted using a fan-assisted extraction system as a sample preparation technique.

2. Development and optimization of greener methodologies for sample preparation

This line of investigation addressed sustainability at the sample preparation stage through the development of miniaturized and environmentally improved extraction procedures for volatile compounds in food matrices.

The specific goals included:

- Optimization of ultrasound-assisted DLLME strategies through the reduction of solvent volumes and selection of greener extraction solvents for the extraction of carbonyl compounds from adult nutritional formulas and coffee samples.
- Assessment of environmental sustainability of the developed DLLME methodology using a greenness evaluation tool, the AGREEprep metric.

Thesis Layout

This thesis is structured in 4 parts, briefly described in the following paragraphs.

Part A is divided into two chapters. Chapter A 1 presents general aspects of coffee (the main food matrix studied in this thesis) and the key factors in the coffee production chain that affect its chemical composition. Conventional methodologies used for the determination of key groups of compounds in coffee are also described in this chapter, along with a discussion of their main advantages and limitations. An overview of green chemistry and its importance in the development of nowadays analytical methodologies is also provided. Chapter A 2 focuses on low-pressure chromatographic flow systems, presenting an extensive critical review of flow injection techniques featuring chromatographic columns. The critical experimental parameters influencing the analytical performance of these systems are presented and discussed according to: mobile phase propulsion devices, sample injection strategies, columns typology, detection systems, data acquisition, selectivity, chromatographic performance, and case-studies. This chapter was adapted from a review published in a peer-reviewed scientific journal.

Parts B and C address the experimental results of this doctoral research. Each part is divided in two chapters, one for each experimental work performed, all adapted from original research articles published in peer-reviewed scientific journals.

Part B focused on the application of low-pressure chromatographic flow systems with electrochemical detection. Chapter B 1 studied the parallel determination of oxidizable and reducible compounds in a single chromatographic run through multiple pulse amperometry. The analytical system featured a 1-cm length C_{18} monolithic column to enable the chromatographic separation of trigonelline, caffeine, and the main chlorogenic acids occurring in coffee. Chapter B 2 focused on the application of a similar system, now with a 0.5-cm length phenyl monolithic column, for the determination of volatile α-dicarbonyl compounds in coffee samples. In this chapter, studies exploring the applicability of the fan-assisted extraction system with derivatization for the extraction of the target compounds, prior to chromatographic analysis, are also presented. The optimization steps performed for chromatographic separation and electrochemical detection are described in detail.

Part C focused on sustainable sample preparation methods based on ultrasound assisted liquid-liquid microextraction. Chapter C 1 studied on the application of an

established sample preparation strategy for the determination of carbonyl compounds in adult nutritional formulas. This chapter describes the optimization of extraction parameters, including time and temperature, as well as the assessment of carbonyl variations in carbonyl composition during sample storage under different conditions. Chapter C 2 focused on a greener approach to the previous methodology for the determination of the same class of compounds in coffee samples. This chapter described the use of greener solvents and the experimental design approaches employed to optimize the conditions affecting the sample preparation methodology.

Part D presents the conclusions derived from the studies conducted, critically discussing the main results obtained from the low-pressure chromatographic flow systems and sample preparation studies. Future work and perspectives are also discussed in this final part.

PART A

Introduction

Chapter A 1

Coffee: Generalities and Analytical Methods

A 1.1. An Overview of Coffee

Coffee is one of the most widely consumed beverages in the world, appreciated for its stimulating effects and complex sensory properties [1]. It is estimated that around 2 billion cups of coffee are consumed daily [2]. Coffee consumption in Europe accounts for over 30 % of the global coffee production [3], making it the continent with the largest coffee market in the world. In contrast, South America is the largest coffee-production region, with Brazil leading the global production and exports [4]. The increasing demand for coffee and the growing preference for high-quality coffee products have contributed to the increased pressure on the producers and coffee roasters to meet these demands [4].

In coffee taxonomy, the term *species* refers to the biological classification (like *Coffea Arabica* and *Coffea Canephora*), while *variety* refers to the naturally occurring populations within a *species*, such as *Coffea Arabica* var. *typica* or *Coffea Canephora* var. *robusta,* or to cultivated variants resulting from human intervention [5]. The term *cultivar* is also accepted, as it is a shortening of 'cultivated variety' [6].

The most predominant species of coffee in the market are *Coffea Arabica* (Arabica) and *Coffea Canephora* var. *robusta* (Robusta) [7], accounting for approximately 61 % and 39 % of the world production, respectively [8]. Arabica is the most appreciated one, presenting a richer sensory profile, characterized by a finer, more aromatic and pronounced flavour [9,10], with sweet and fruity notes [11]. Robusta is known for its bitterness and enhanced body and crema formation in the beverage [10]. The cultivation of Arabica coffee is associated with higher costs due to its more demanding environmental and harvesting conditions, and coffee processing, especially when focusing in obtaining a high quality coffee [10,12]. In contrast, Robusta coffee is more tolerant to climate changes [13], less demanding regarding cultivation conditions, and more resistant to pests and diseases [14]. Based on the abovementioned scenario, coffee roasters normally commercialize blends of Arabica and Robusta with proportions optimized for desired flavour profiles [10], while reducing the final cost of coffee production to the consumer. However, some brands mislabel their blended coffee as 100 % Arabica or claim higher proportions of Arabica coffee to increase profit [11]. Although these two species present very distinct organoleptic properties when prepared individually, small amounts of Robusta may remain undetected in blends, as even experienced tasters can estimate the ratio of the blend with an error of approximately 20

% [15]. This type of fraud is a concern in coffee authentication and poses a significant analytical challenge.

The quality of coffee beans is assessed using a 100-points grading scale, where beans scoring 80-100 points are classified as specialty coffee, and those scoring below 80 points are categorized as commercial coffee [16]. This classification is a key determinant of coffee value. However, coffee price depends not only on this classification, but also in other factors within the coffee chain, such as producers, wholesale and exportation prices [16]. The growing demand for high quality coffee and the increasing interest in specialty coffee have led to an increase in research focused on the factors that influence beverage quality [4,14]. Coffee quality results from a complex chain of factors, ranging from cultivation to beverage preparation [17], as presented in Figure 1. At each step, the chemical composition of the coffee bean is influenced, affecting not only the aroma and taste, but the overall sensory experience and nutritional properties of the beverage [4,16,18]. The main aspects associated with each stage are briefly discussed below.

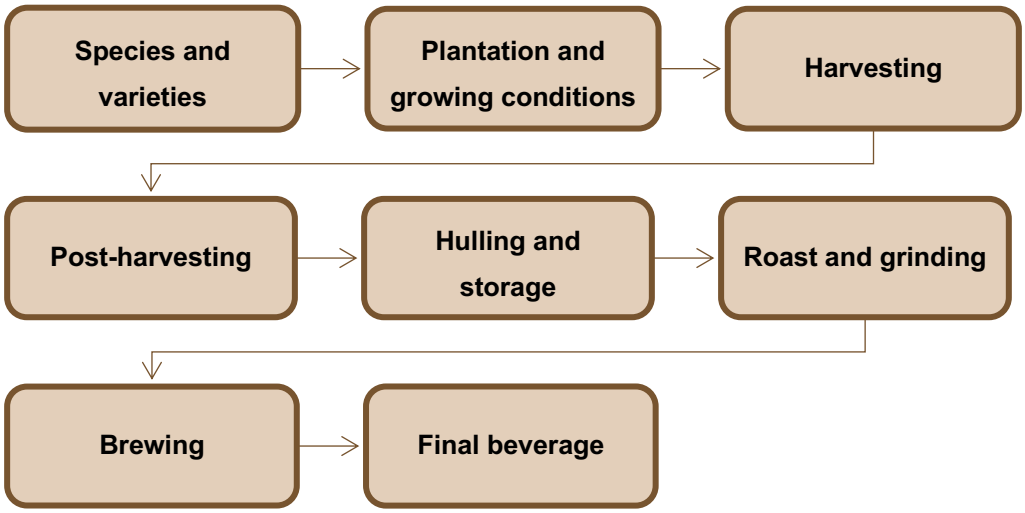

Figure 1 – Production chain of coffee, from selection of coffee species to preparation of the final beverage.

The coffee quality journey starts with the selection of the coffee species and variety. The geographic location, climate, and desired organoleptic properties are factors considered when selecting the coffee plant [19]. The respective genetic predisposition defines inherent quality traits of coffee [19]. Robusta coffee beans are richer in caffeine and chlorogenic acids (CGAs) (contributing to coffee's bitterness), while Arabica coffee

beans present higher concentrations of trigonelline and lipids [8]. Cultivars such as Arabusta (Arabica-Robusta hybrid) were created as a cost-quality alternative, with the goal of producing coffee with higher quality than Robusta while being more vigorous and disease-resistant than Arabica [20].

Most coffee producers begin by germinating seeds in nurseries before transferring them to the production field. This process normally takes between 6 to 12 months [6]. After transplantation, factors such as altitude, soil, temperature, rainfall and sunlight exposure influence the chemical composition and sensory attributes of coffee [17,21,22]. The ideal conditions for the coffee plant growth are tropical zones near the equatorial zone known as “The Coffee Bean Belt” [23]. Examples of the plantation conditions impact upon the coffee chemical composition are reported in the literature. Hu et al. [24] reported a positive correlation between higher plantation altitude and coffee chemical composition and quality in Pu’er City; Pappo et al. [25] reported that soil moisture influences volatile compounds and sensory properties of coffee, depending on the selected cultivars.

The coffee tree will take about 3 to 5 years of growing for the fruit to be qualified for coffee production and will be in full bearing after 6 to 8 years [26]. Thereafter, most coffee trees have one harvest *per* year, with some regions presenting the possibility of a second harvest due to climatic conditions. However, this secondary harvest is generally smaller, and beans will likely present lower quality [6]. Harvest must be done once the coffee cherries are fully mature, normally between 7 to 9 months after flowering [26], to ensure optimal flavour [20]. The harvesting process is usually performed by strip (manually or mechanically) or selective picking [27], and significantly influences the uniformity and quality of the coffee crop [28]. Since coffee maturation is not uniform, strip picking may include under-ripe and over-ripe cherries, negatively affecting flavour, while selective picking, although labour-intensive, enables the harvesting of predominantly fully ripe cherries.

At the post-harvesting stage, the aim is to remove the skin and pulp of the cherry (Figure 2), normally by one of three processing methods: dry, semi-dry or wet [27]. These methods influence acidity, body and flavour of coffee [29]. In the dry or “natural” method, cherries are dried under sun exposure and/or resourcing to air dryers until a 10-12 % (w/w) of moisture content is obtained [30]. The dried skin and pulp are then removed mechanically or manually [31]. This process typically lasts 3 to 5 weeks. In the wet method, the coffee cherries are washed and the pulp removed mechanically (using a pulper), followed by mucilage removal which can be performed either by chemical/enzymatic products or through natural fermentation. The green coffee beans

are then washed and dried [32]. This process typically reduces processing time from weeks (dry process) to 8-10 days (wet process) [30]. The semi-dry, or honey, method combines elements of both previous methods. The cherries initially undergo wet processing, followed by a drying step until moisture content of 10-12 % w/w is achieved [33]. Dry methods generally result in coffees with lower acidity and more complex flavour profiles; whereas wet methods yield fruity beverages with lighter and cleaner characteristics; semi-dry retains mucilage during drying, producing intermediate flavour profiles [32]. Selection of coffee cherries can also be performed during post-harvesting processing, particularly during washing and/or drying steps, to remove under-ripe and over-ripe cherries.

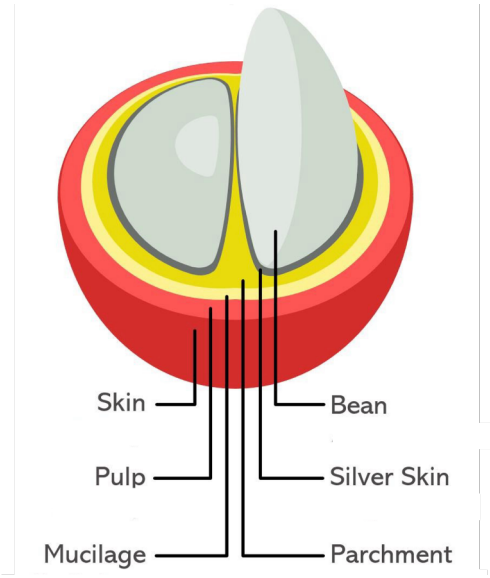

Figure 2 – Cross-section view of the coffee cherry showing the layers, adapted from Gilberto V. de Melo Pereira et al. [30].

After drying of the coffee beans, these are normally stored for a period of 30 to 60 days [6]. Thereafter, coffee beans are hulled (to remove the parchment and silver skin) and sorted according to size, colour and density [34]. This step can be performed mechanically or manually (the latter being more common) aiming to remove defective beans, ensure size uniformity [31,34,35] and enable grading [6]. Green coffee beans are then ready to be bagged. To increase their shelf-life, coffee beans must be stored under controlled conditions of humidity and temperature [34]. In some cases, storage bags are lined with a protective layer to avoid moisture, thereby ensuring safer storage and transportation [6]. When in protective bags, green coffee beans can be stored up to 3

years, under ideal conditions (approximately 11 % of humidity, low temperature and inert atmosphere to prevent oxidation) [30], however, these ideal conditions, especially the inert atmosphere, add cost to the final products. Nonetheless, shorter storage periods are recommended to not impact beverage quality [30].

The roasting process is one of the final steps before coffee brewing. During roasting, temperature-dependent chemical reactions occur, including Maillard reactions, carbohydrate caramelization, Strecker degradation and oxidation reactions [36,37]. The roasting degree of the coffee beans defines the extent of these reactions, and therefore the compounds formed, which influence the sensory properties of the final beverage [38]. Green coffee beans can be roasted to various degrees. In general terms, coffee roast intensity can be classified as light (associated with sweet and nutty aromas), medium (complex aroma profile) or dark roast (burnt, sour and ashy aromas) [39]. These changes in the coffee's chemical composition contribute to the complexity of coffee analysis due to the wide diversity of compounds formed. The packaging of coffee after roasting is also a main impact factor in coffee shelf life and quality. Unsealed craft packaging (using craft paper bags) is a cheaper choice when the coffee is intended to be consumed days after roasting. Sealed foil packing (foil bags with a valve to prevent fresh air from entering and allow the carbon dioxide to exit) adds a cost to the final product but increase the coffee shelf life. Gas-flushed sealed foil packaging (foil bags flushed with an inert gas) is the option that offers the longer shelf life to coffee, since the inert atmosphere helps preventing oxidation reaction to occur and helps preserving the aromatic compounds formed during the roasting process [6]. The use of an inert gas, as previous mentioned, increases the cost of the final product.

After roasting, coffee beans are ground to obtain the coffee powder used for beverage preparation. During brewing, the aromatic compounds developed during the roasting process are extracted into the final beverage [40]. Depending on the type of coffee and the desired beverage (flavour profile and brewing method), coffee grinding can be divided into 4 categories: very fine, fine, medium and coarse [6]. Consistency in coffee powder size is also important for coffee cup quality. Grinding should be performed as close as possible to the brewing stage to minimize exposure to air and prevent quality degradation.

Brewing is the final step in coffee preparation, and beverage quality depends not only on the coffee but also on the chemical composition of the water used. Although often overlooked, water properties, such as hardness and mineral content, significantly influence the flavour of the beverage [6]. If the water is too hard (high concentrations of

minerals, salts and metals), it may lead to under-extraction of compounds from the coffee powder, conversely, if the water is too soft (low content in minerals and salts), it may result in over-extraction, often resulting in undesirable flavours [5]. Brewing procedures are generally classified into three main categories: decoction (e.g., boiled and Turkish coffee), infusion (e.g., filter coffee) and pressure (e.g., espresso and moka coffee) [41]. Decoction methods involve prolonged contact between coffee grounds and hot water, allowing extensive extraction of soluble compounds. Infusion methods rely on the percolation of hot water through ground coffee. Pressure methods use pressurized hot water to pass through compacted coffee grounds. Different brewing approaches result in differences in the chemical composition of the final beverage and, consequently, its organoleptic properties.

A 1.2. Chemical Complexity, Quality, and Traceability in Coffee Analysis

Coffee's complex composition depends on the coffee species, variety, as well as on all processing stages that occur after harvesting. Each of the processing stages described in the previous section promotes changes in the chemical composition of coffee, which directly influence the sensory attributes, nutritional profile, and potential health effects of the final beverage [42–44].

Among the main compounds present in coffee are caffeine, the most abundant alkaloid in coffee, responsible for its stimulating properties and potential neuroprotective effects [45,46]; trigonelline, which contributes to the bitterness of coffee and is a precursor of aroma compounds during roasting [47,48]; and phenolic compounds, particularly CGAs, which are associated with antioxidant, anti-inflammatory and antidiabetic properties [49]. During roasting, CGAs also contribute to the formation of aroma compounds and Maillard reaction products that impact bitterness and colour, namely melanoidins [32]. Furthermore, the lipid fraction, which includes diterpenes such as cafestol and kahweol, has been linked to both potential anticarcinogenic and hepatoprotective effects [50,51], as well as with increased cholesterol levels. A summary of the main compounds occurring in green and roasted coffee is given in Table 1.

Table 1 – Key compounds in Arabica and Robusta coffee and their concentration in green and roasted beans. Concentrations are presented in g / 100 g of coffee in dry weight basis.

Compounds	Arabica		Robusta		Ref
	Green	Roasted	Green	Roasted	
Polysaccharides	50-55	24-39	37-47	41.5	[52,53]
Sucrose	7.4-11	----	4-7	----	[54,55]
Lipids	12-18	14.5-20	9-13	11-17	[52]
Proteins	8.5-13	13-15	11-13	10	[52,53]
Minerals	3-4.2	3.5-4.5	4-4.5	4.5-4.7	[52,53]
Chlorogenic acids	5.5-8	1.2-2.3	7-10	2.7-3.1	[52,53]
Caffeine	0.8-1.4	1.3	1.5-2.4	2.4	[53,56]
Trigonelline	0.9-1.8	0.1-1	0.7-1.2	0.1-0.9	[52,54,57]
Melanoidins	----	16-17	----	23	[52,53]

The chemical complexity of coffee makes the development of reliable analytical methods essential to control coffee quality. For this reason, the development of analytical methodologies is a valuable tool to monitor chemical compositional changes in coffee throughout the production chain, and to evaluate quality consistency across different batches from plantation to the final beverage [42–44].

In this scenario, analytical chemistry plays a crucial role in both traceability and authentication assessment of coffee. Traceability refers to the ability to track coffee through all stages of the production chain, from plantation to retail, ensuring that all information is preserved and accessible [58,59]. It is particularly important in specialty coffee markets, where origin and production conditions are strongly associated with quality perception and economic value [60]. Authentication, in turn, addresses the growing need to verify product integrity in the coffee market. As previously mentioned, the most common form of fraud in the coffee market is the adulteration of coffee labelled as 100 % Arabica with blends of Arabica and Robusta coffee. In this context, the development of analytical methodologies capable of discriminating between both species and verifying product labelling is of utmost relevance [61,62]. Together, traceability and authentication contribute to consumer trust, market transparency and the long-term sustainability of coffee production and trade [58].

Within the broad chemical diversity of coffee composition, this thesis focuses on the following compounds of coffee: caffeine, trigonelline, CGAs, and carbonyl compounds. These analytes were chosen because they are key chemical compounds directly linked to quality, health relevance, and authenticity. They also typify the methodological challenges associated with coffee analysis, since abundant compounds such as caffeine and trigonelline require robust yet rapid analytical methodologies for quantification, while the characterization of CGAs requires high selectivity due to the structural similarity between these compounds. Lastly, volatile carbonyl compounds occur at low concentrations and require both highly sensitive analytical techniques and sample preparation strategies based on extraction and/or derivatization steps.

In this context, the following section briefly reviews existing analytical methodologies for the determination of these compounds, evaluating their advantages and limitations. Furthermore, it is discussed the growing importance of green analytical strategies, addressing the increasing demand for environmentally sustainable analytical methodologies.

A 1.3. Current Trends in Coffee Analysis

The analytical methodologies published in the past two decades for the determination of the target analytes in this thesis (trigonelline, caffeine, CGAs, carbonyl compounds) are presented in the following four tables. This state-of-art illustrates the current diversity of methodologies normally used for this purpose. A brief summary and discussion are presented in this context.

Table 2 – Examples of analytical methodologies used for the determination of caffeine in coffee samples.

Year	Methodologies			Sample	LOD	LOQ	Observations	Ref
	Sample preparation	Chromatographic technique	Detection					
2000	Extraction with NH_3 LLE with $CHCl_3$	n.a.	Spectrometry (FTIR)	Roasted coffee	3 mg/L	n.p.	5 mL of $CHCl_3$ *per* extraction	[63]
2008	Extraction in hot water Filtration Cleanup step: SPE (MeOH and $CHCl_3$)	LC C_8 column (150 x 4.6 mm, 5 µm)	Spectrophotometry (DAD)	Roasted coffee	0.07 mg/L	0.2 mg/L	Isocratic elution (90:10 (v/v) – 0.1 % THF in water, pH 8 : ACN) 0.64 mL of ACN *per* run 12 mL of MeOH, 14 mL of ACN and 10 mL of $CHCl_3$ *per* extraction	[64]
2008	Extraction in hot water Cleaning step (basic lead acetate solution) Filtration Dilution prior to analysis	LC C_{18} (150 x 2.0 mm, 5 µm)	Spectrometry (MS with ESI)	Green and roasted coffee	1.2 mg/100 g	4.0 mg/100 g	Gradient elution (0.3 %, v/v aqueous HCOOH and MeOH) < 1 mL of MeOH *per* run	[65]
2012	Extraction in hot water Decantation Dilution	n.a.	Electrochemistry (SWV) E_w – modified GCE	Roasted coffee	0.137 µmol/L	------	No organic solvent used	[66]
2014	Ultrasound assisted LLE (water and MeOH)	LC C_8 column (125 x 4.6 mm, 5 µm)	Spectrophotometry (UV-Vis detector)	Roasted and green coffee	1.90 µg/L	5.70 µg/L	Gradient elution (1 % v/v H_3PO_4 in water and 1 % v/v H_3PO_4 in ACN) < 8 mL of ACN *per* run < 24 mL of MeOH *per* extraction	[67]
2017	Extraction in hot water Decantation	n.a.	Electrochemistry (DPV) E_w- Lignin modified GCE	Roasted coffee	0.837 µmol/L	2.79 µmol/L	No organic solvent used	[68]
2021	Salting-out assisted LLE	n.a.	Spectrophotometry (UV-Vis detector)	Green coffee	0.96 mg/L	2.86 mg/L	Partition of analytes in LLE: Caffeine – ACN phase 12 mL of ACN *per* extraction	[69]
2023	Extraction with hot water Filtration Dilution prior to analysis	LC C_{18} column (150 x 4.6 mm, 3 µm)	Spectrophotometry (DAD)	Roasted coffee	0.059 mg/L	0.178 mg/L	Gradient elution (5 % v/v acetic acid in water and ACN) < 1.5 mL of ACN *per* run	[70]
2025	Extraction in hot water Dilution Centrifugation Filtration	LC C_{18} column (100 x 4.6 mm, 3.5 µm)	Spectrophotometry (DAD)	Roasted coffee	0.44 mg/L	1.76 mg/L	Gradient elution (0.1 % v/v H_3PO_4 in water and MeOH) < 3 mL of MeOH *per* run	[71]

LOD – Limit of Detection / LOQ – Limit of Quantification / n.a. – non-appliable / n.p. – not presented / ESI – Electrospray ionization / DPV – differential pulse voltammetry / E_w – Working electrode / FTIR – Fourier transform infrared spectroscopy / SWV – Square wave voltammetry / THF – Tetrahydrofuran

Table 3 – Examples of analytical methodologies used for the determination of trigonelline in coffee samples.

Year	Methodologies			Sample	LOD	LOQ	Observations	Ref
	Sample preparation	Chromatographic technique	Detection					
2008	Extraction in hot water Cleaning step (lead acetate solution) Filtration Dilution prior to analysis	LC C_{18} column (150 x 2.0 mm, 5 µm)	Spectrometry (MS with ESI)	Green and roasted coffee	3.6 mg/100 g	12.1 mg/100 g	Gradient elution (0.3 % v/v HCOOH in water and MeOH) < 1 mL of MeOH *per* run	[65]
2015	Extraction (H_2O and MeOH) assisted with ultrasound Centrifugation Supernatant filtration	LC C_{18} column (150 x 4.6 mm	Spectrophotometry (UV-Vis detector)	Roasted coffee	0.3 µmol/L	n.p.	Ion pair rent chromatography Isocratic elution (0.1 % v/v H_3PO_4, 4 mM octanesulfonate and 15 % v/v MeOH) 1.5 mL of MeOH *per* extraction 3.45 mL of MeOH *per* run	[72]
2021	Salting-out assisted LLE	n.a.	Spectrophotometry (UV-Vis detector)	Green coffee	1.51 mg/L	4.58 mg/L	Partition of analytes in LLE: Trigonelline – aqueous phase 12 mL of ACN *per* extraction	[69]
2023	Extraction with hot water Filtration Dilution prior to analysis	LC C_{18} column (150 x 4.6 mm, 3 µm)	Spectrophotometry (DAD)	Roasted coffee	0.047 µg /mL	0.138 mg/L	Gradient elution (5 %v/v acetic acid in water and ACN) < 1.5 mL of ACN *per* run	[70]

n.a. – non-appliable / n.p. – not presented

Table 4 – Examples of analytical methodologies used for the determination of CGAs in coffee samples. The presented values of LOD and LOQ are only related to 5-caffeoylquinic acid (5-CQA), the most abundant isomer of CGAs in coffee.

Year	Methodologies			Sample	LOD	LOQ	Observations	Ref
	Sample preparation	Chromatographic separation	Detection					
2008	Ultrasound assisted LLE (water and MeOH)	LC C_8 column (125 x 4.6 mm, 5 µm)	Spectrophotometry (UV-Vis detector)	Roasted and green coffee	1.65 µg/L	5.00 µg/L	Gradient elution (1 % v/v H3PO4 in water and 1 % v/v H3PO4 in ACN) < 8 mL of ACN *per* run < 24 mL of MeOH *per* extraction	[65]
2016	Extraction (2 % v/v formic acid and 10 % ACN) assisted with sonication Filtration	LC C_{18} column (100 x 4.8 mm, 5 µm)	Spectrophotometry (UV-Vis detector)	Green coffee	n.p.	1.25 mg/L	Appliable for the quantification of other 6 isomers of CGAs in green coffee Gradient elution (0.1 % v/v TFA in water and ACN) 4 mL of ACN *per* extraction < 9 mL of ACN *per* run	[73]
2016	Extraction in hot water Filtration	n.a.	Electrochemistry (DPV) E_W - GCE	Green and roasted coffee	1.2 µmol/L	4 µmol/L	Appliable for the characterization of other 8 isomers of CGAs in coffee Determination of the total CGAs content in coffee No organic solvent used	[74]
2020	Extraction in hot water Centrifugation Filtration	LC C_{18} column (100 x 2.1 mm, 1.7 µm)	Spectrometry (triple quadrupole mass spectrometer with ESI source)	Green coffee	0.016 mg/L	0.051 mg/L	Gradient elution (0.1 % v/v HCOOH in water and ACN) 2 mL of ACN *per* run	[75]
2021	Salting-out assisted LLE	n.a.	Spectrophotometry (UV-Vis detector)	Green coffee	0.879 mg/L	2.66 mg/L	Determination of only 5-CQA from CGAs Partition of analytes in LLE: 5-CQA - ACN phase 12 mL of ACN *per* extraction	[69]
2023	Extraction with hot water Filtration Dilution prior to analysis	LC C_{18} column (150 x 4.6 mm, 3 µm)	Spectrophotometry (DAD)	Roasted coffee	0.017 mg/L	0.052 mg /L	Gradient elution (5 % v/v acetic acid in water and ACN) < 1.5 mL of ACN *per* run	[70]

n.a. – non-appliable / n.p. – not presented / DPV – differential pulse voltammetry / ESI – Electrospray ionization / E_w – Working electrode

Table 5 – Examples of analytical methodologies used for the determination of α-dicarbonyl compounds (α-DC) in coffee samples.

Year	Methodologies			Sample	LOD	LOQ	Observations	Ref
	Sample preparation	Chromatographic separation	Detection					
2014	Extraction in hot water Filtration Clean-up step: SPE (MeOH and H_2O) Derivatization (OPDA) SPE (MeOH:H_2O , 90:10 v/v)	LC C_{18} column (150 x 2.1 mm, 5 µm) Guard column C_{18} (10 x 2.1 mm, 5 µm)	Spectrophotometry (DAD)	Roasted coffee	0.01 mg/L (GO) 0.01 mg/L (MGO) 0.002 mg/L (DA)	0.02 mg/L (GO) 0.03 mg/L (MGO) 0.006 mg/L (DA)	Determination of α-DC compounds Gradient elution (0.1 % v/v HCOOH in water and MeOH) 13.6 mL of MeOH *per* extraction < 12 mL of MeOH *per* run	[76]
2018	GDME with derivatization (DNPH)	LC C_{18} column (150 x 4.6 mm, 3 µm) Guard column (4.0 x 3.0 mm)	Spectrophotometry (DAD)	Roasted coffee	n.p	n.p	Gradient elution (ACN and acetate buffer) 250 µL of ACN *per* extraction < 20 mL of ACN *per* run	[77]
2021	2 extraction methods: Espresso and cold brewing Filtration LLE (H_2O and ethyl acetate) with derivatization (OPDA)	GC DB-WAX column (30 m x 0.25 mm i.d., 0.25 µm film thickness)	Spectrometry (NPD)	Roasted coffee	0.1 mg/L (GO) 0.08 mg/L (MGO) 0.16 mg/L (DA)	0.33 mg/L (GO) 0.25 mg/L (MGO) 0.5 mg/L (DA)	Determination of α-DC compounds Helium was used as carrier gas (1.5 mL/min) 5 mL of ethyl acetate *per* extraction	[78]
2021	2 extraction methods: Espresso and cold brewing SPME (DVB/CAR/PDMS fibber)	GC DB-WAX column (60 m x0.25 mm i.d., 0.25 µm phase thickness)	Spectrometry (MS)	Roasted coffee	n.p	n.p	Helium was used as carrier gas (1.0 mL/min) Applied in the determination of 24 volatile compounds in coffee	[79]
2022	Extraction with hot water SPME (CAR/PDMS fibber)	GC ZB-FFAP column (60 m x 0.25 mm i.d., 0.25 µm)	Spectrometry (triple quadrupole mass spectrometer with ESI source)	Roasted coffee	n.p.	n.p.	Helium was used as carrier gas (1 mL/min) Applied to the monitorization of 21 volatile compounds of coffee	[80]

n.a. – non-appliable / n.p. – not presented / DVB/CAR/PDMS – Divinylbenzene/carboxen/polydimethylsiloxane / ESI – Electrospray ionization / NPD – Nitrogen-phosphorus detector

For the determination of caffeine, trigonelline, and CGAs, liquid chromatography (LC) combined with spectrophotometric, or mass spectrometric detectors are the most widely used analytical techniques due to their robustness, reproducibility, and ability to perform multi-analyte determination. Reverse-phase chromatography, employing C_{18} and C_8 stationary phases is commonly used. In particular, mass spectrometry detection [75] remains essential for the accurate identification of CGAs isomers, due to their similar structure and absorbance spectra.

Recent developments indicate a gradual reduction in solvent consumption *per* run, reflecting the growing awareness of green analytical chemistry principles. Nevertheless, LC-based methodologies remain inherently dependent on the consumption of organic solvents and relatively complex instrumentation, limiting their sustainability and accessibility, particularly due to the initial investment required.

The determination of carbonyl compounds – especially α-dicarbonyl compounds – relies predominantly on gas chromatography (GC). This technique, combined with SPME, offers the advantage of being solvent-free [79,80]. When LC based strategies are employed, a derivatization step is typically performed to enhance compound stability and detectability. Derivatization reagents such as ortho-phenylenediamine (OPDA) [76] and 2,4-dinitrophenylhydrazine (DNPH) [77] improve selectivity but introduce additional reagents and experimental steps into the workflow. Sample preparation may include solid-phase extraction (SPE) as a clean-up procedure [76,77] increasing solvent consumption. A recent sample preparation methodology, gas-diffusion microextraction (GDME) [77], uses reduced volumes of extracting solvents, illustrating the increasing emphasis on environmental sustainability in analytical method development.

Analytical methodologies without chromatographic separation based on electrochemical techniques, as square wave voltammetry [66], and differential pulse voltammetry [68], have been proposed for the determination of caffeine and CGAs. These methods offer simplified instrumentation and reduced consumption of organic solvents. However, their selectivity is limited, due to potential matrix interferences. As a result, they are often restricted to single-analyte or total-content determinations, such as the total CGA content in coffee, as shown in Table 4.

Similarly, spectrophotometric approaches [69] (without chromatographic separation) offer operational simplicity but lack the required selectivity for comprehensive compound profiling.

Across all analytes considered, a consistent trade-off emerges, chromatographic methods provide selectivity and multi-analyte determination capability at the expense of

higher solvent and energy consumption, and increased cost (particularly in terms of initial investment), whereas simpler techniques reduce environmental impact but compromise analytical resolution.

Despite the notable progresses made towards greener protocols, based on reducing solvent volume consumption, miniaturization of the extraction techniques, and improving detection sensitivity, a gap remains in current developments. Few methodologies simultaneously achieve reduced solvent consumption, simplified instrumentation, multi-analyte capability, and adequate selectivity in complex matrices. Addressing this challenge is the central rationale of this thesis.

A 1.4. Green Strategies in Coffee Analysis

In recent years, the development of greener analytical approaches has become increasingly important. Conventional methodologies, although robust and accurate, often rely on toxic solvents, high energy consumption processes, and generating high amounts of waste. Green approaches, in line with the principles of Green Analytical Chemistry, aim to minimize the environmental footprint of analytical processes by reducing and/or avoiding solvent consumption, replacing hazardous solvents with safer and greener alternatives, improving energy consumption, and adopting solvent-free or miniaturized analytical systems [81].

A 1.4.1. Green Solvents

Green solvents have attracted increasing attention as sustainable alternatives to conventional organic solvents for the extraction of compounds from different food matrices. Their growing relevance aligns with global efforts to minimize environmental impact and support greener analytical practices.

The categorization of solvents is performed using selection guides that evaluate the impact of a solvent in different parameters (such as, acute toxicity, biodegradation, and global warming potential). However, different classifications have been proposed according to different organisations so that solvent categorization is, therefore, not yet consensual. Byrne et al. [82] presented a categorization table for solvents, according to 3 guides from biopharmaceutical companies, respectively, Pfizer, GSK and Sanofi. A good example of categorization inconsistence is the classification of ACN, one of the most frequently used organic solvents in conventional methods, due to its strong elution

power and high compatibility with reverse-phase chromatography [83], but associated with safety hazards to human health [84]. As shown in Table 6, ACN was categorized as yellow (i.e. usable) according to Pfizer, red (major issues) according to GSK, and green (recommended) according to Sanofi. Regarding solvents with green categorization, water, 1-butanol and *i*-propyl acetate are the only ones in all 3 guides. Following this categorization table, ethyl acetate, dimethyl sulfoxide and some alcohols (MeOH, EtOH, 1-propanol and *i*-propanol) are classified as green in 2 of the guides and yellow in one of them.

Table 6 – Examples of solvent categorization according to 3 guides (Pfizer, GSK and Sanofi), adapted from Byrne et al. [82].

Class	Solvent	Classification		
		Pfizer	GSK	Sanofi
Alcohols	Methanol (MeOH)	Preferred	Some issues	Recommended
	Ethanol (EtOH)	Preferred	Some issues	Recommended
	1-Propanol	Preferred	Some issues	Recommended
	i-Propanol	Preferred	Some issues	Recommended
	1-Butanol	Preferred	Few issues	Recommended
Dipolar Aprotic	Dimethyl sulfoxide	Usable	Some issues	Substitution advisable
	Acetonitrile (ACN)	Usable	Major issues	Recommended
	Dimethylformamide	Undesirable	Major issues	Substitution requested
Esters	Ethyl acetate	Preferred	Some issues	Recommended
	i-Propyl acetate	Preferred	Few issues	Recommended
Halogenated	Dichloromethane	Undesirable	Major issues	Substitution advisable
	Chloroform	Undesirable	Major issues	
Hydrocarbons	Benzene	Undesirable	Major issues	
	Isooctane	Usable	Some issues	
	Toluene	Usable	Some issues	Substitution advisable
Miscellaneous	Water	Preferred	Few issues	Recommended

Note: Black entries indicate solvents that should be banned according to the respective guide. White entries indicate lack of sufficient information to assign an adequate classification to the solvent according to the guide.

Beyond conventional green solvents, significant interest has emerged in next-generation alternatives such as deep eutectic solvents (DES). DES are tailored solvent systems, formed by the interaction between hydrogen bond acceptors and hydrogen bond donors [85], through the mixture of two or more eco-friendly components [86]. Quaternary ammonium salts with hydrogen bond donors, such as amides, alcohols, and carboxylic acids are common components of DES [87]. A key advantage of DES is their tunability: by selecting appropriate components, DES can be designed to selectively

solubilize specific classes of compounds, potentially improving extraction efficiency and selectivity [88,89].

The main area of application for DES is the metal finishing industry. Current processes in this area are based on aqueous solutions, which present a limited potential window. Therefore, DES have been studied as alternatives for metal deposition, dissolution and processing, offering a wider potential working range, among other advantages [89]. Even though numerous scientific papers and reviews have been published over the past 20 years, scaling up demonstrations remain limited [90]. Recently, DES have also been applied in food matrices, as demonstrated by Qingyue et al. [91], where DES were optimized to increase the extraction of zearalenone from several food matrices.

Despite these promising features, DES are a controversial green alternative, since recent studies indicate that some DES may be less environmentally friendly than initially assumed, and their toxicity requires further evaluation, as conventional toxicity tests are not always suitable due to the high viscosity of DES [92].

Green solvents have already been successfully implemented in the extraction and separation of coffee bioactive compounds, such as CGAs, caffeine, and phenolic compounds. Pishro et al. [93] studied hydrophobic DES as alternative to traditional organic solvents for the extraction of caffeine from coffee beans, and coffee skin. Silva et al [94] explored the application of hydrophilic and hydrophobic DESs in the extraction of bioactive compounds (caffeine and chlorogenic acids) from coffee by-products, such as coffee skin and pulp, in order to facilitate the use of coffee waste without generating further solvent waste. Oliveira et al. [95] evaluated the application of ethanol, acetone, ethyl acetate, among other solvents, for the extraction of bioactive compounds (focusing on phenolic and antioxidant compounds) from green coffee beans to promote the valorisation of coffee waste.

A 1.4.2. Microextraction strategies and green approaches

Miniaturization and solvent consumption reduction are among the primary strategies for improving the sustainability of analytical extraction methods leading to the development of microextraction strategies. Some most widely known examples of microextraction strategies are solid-phase microextraction (SPME), and dispersive liquid-liquid microextraction (DLLME). Additional approaches include gas diffusion microextraction (GDME), and fan-assisted extraction (FAE). All these analytical

strategies present lower consumption of solvents compared to conventional liquid-liquid extraction (LLE) procedures [77,96,97].

SPME is a solvent-free technique that allows the sampling, extraction and concentration of analytes by using a small fused silica fiber, coated with an adsorbent material [98]. The fiber is mounted in a syringe-like device suitable to pierce a septum closed flask containing the sample to be analysed. The fiber is then exposed in the headspace and the volatile compounds are adsorbed on to the fiber for a defined period. After extraction, the desorption of the compounds in the fiber is performed in the injector of the gas chromatograph [99].

GDME approach was developed in the same research group as this thesis (QuAQuA), and it presents a combination of microextraction with membrane-aided gas diffusion [100]. GDME is based on a tube featuring a volatile compound permeable membrane fixed transversally in its middle. The tube is assembled in the cap of a gas-tight flask containing the sample. In the upper face of the membrane, a defined volume of an acceptor solution is poured. The membrane allows the volatile compounds to pass through it, so that the volatile compounds are trapped in the acceptor solution. The acceptor solution is thereafter analysed by chromatography.

FAE approach was the latest extraction strategy developed in QuAQuA group, to be applied for volatile compounds. This technique relies on a closed flask featuring an inner electric fan. In the flask, the sample and an acceptor solution are placed. The convection promoted by the internal electric fan increases the mass transport in the headspace enhancing the extraction yield of the volatile compounds from the sample to the acceptor solution, which is ultimately analysed by high-performance liquid chromatography (HPLC) [101]. FAE was originally applied to green coffee samples [101]. Lately, this approach was also applied to liquid samples, mimicking the full evaporation technique, in the determination of carbonyl compounds in coffee brews [102]. The combination of the strategies FAE and full evaporation technique is the basis of one of the works developed in this thesis and will be explained in more depth in chapter B 2.

DLLME is performed using a ternary solvent system comprising a major aqueous phase (containing the target analytes), a small volume of extraction solvent (immiscible with water), and a dispersive solvent (miscible with both phases) [102]. When the extraction and dispersive solvents are rapidly injected into the aqueous phase sample, a fine cloudy dispersion is formed, creating a large interfacial area between phases, which enables efficient partitioning of analytes into the extraction solvent within a short

time. Following extraction, centrifugation is performed to improve phase separation. The extraction solvent undergoes sedimentation or flotation, depending on its density. The final result is typically a small volume of extraction solvent enriched with the targeted analytes. DLLME thus enables simultaneous extraction and preconcentration in a single rapid step, offering high enrichment factors and excellent analytical precision [103].

DLLME has gained increasing attention due to its simplicity, rapidity, and good analytical performance. Despite its advantages, conventional DLLME often uses toxic chlorinated solvents, such as chloroform ($CHCl_3$) or dichloromethane (CH_2Cl_2), raising environmental and safety concerns [104]. In response, recent developments have focused on greener adaptations of DLLME, replacing these solvents with less hazardous alternatives (such as ionic liquids and long-chain alcohols) and optimizing dispersion using ultrasound or heating rather than conventional dispersive solvents [105–107]. These innovations have enhanced the technique's sustainability and versatility, enabling its application to diverse analytes and complex matrices [108,109].

Miniaturized extraction techniques, such as GDME, DLLME, SPME, and FAE, represent practical strategies for reducing solvent consumption in food analysis. Among these, DLLME is particularly adaptable to different classes of compounds and solvent systems, allowing methodological tailoring according to matrix complexity and sustainability constrains. Recent advances in solvent substitution (using low-toxic solvents) and alternative dispersion strategies further strengthen its sustainability for environmentally conscious analytical workflows. Improvements in the energy efficiency of sample preparation can be achieved by combining DLLME and auxiliary techniques into sample preparation, such as ultrasound-assisted extraction (UAE) and microwave assisted extraction, in a single step [88,110,111].

In sum, green solvents and microextraction techniques are not only environmentally advantageous but also propose analytical reliability, reduced costs, and operational simplicity. They demonstrate the feasibility of integrating green chemistry principles into routine analytical practice. Based on these characteristics, the present thesis is built upon these foundations, using DLLME and related sustainable strategies as part of its experimental framework to develop efficient, reproducible, and environmentally sustainable analytical methodologies for coffee analysis.

Chapter A 2

Feasibility of Low-Pressure Chromatographic Flow Injection Systems: Experimental Concerns and Analytical Applications

This chapter was adapted from the following paper:

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Flow analysis techniques have undergone several developments since the pioneer works from Skeggs et al. [112]. and Ŗužička et al. [113] based on segmented flow analysis and flow injection analysis, respectively. Strong evidence of this scenario is the successive publication of review articles in this area (more than 100) as gathered by Trojanowicz et al. in 2016 [114]. Several fluid management approaches emerged, the most prominent termed flow injection analysis, sequential injection analysis, multicommutation, multisyringe and lab-on-valve. These, together with many ingenious in-line sample treatment developments (e.g. the coupling of reactors, strategies for on-line solid-liquid extraction, leaping detectors), still present limited applicability when multi-component analysis is aimed.

The enhancement of the analytical potentialities of flow analysis systems to conduct multi-component analysis with the feasibility of conventional chromatographic systems, has long been a desired goal. Early works in this context exploited improvised lab-made columns which, despite the inherent prototype limitations (namely, lack of reproducibility and limited separation ability), already constituted a proof-of-concept. An example of such approach is the work of Cox et al, in 1985, who used a column 2.5 cm length, 1.5 mm i.d., filled with activated alumina (particle sizes ranging from 75 to 120 μm) for the determination of Cr(III) and Cr(VI) in water samples using a FIA-ICP-AES manifold [115].

The development of monolithic columns was revolutionary not only in the field of chromatography – as monolithic columns are considered the fourth generation of chromatographic columns [116] – but also in upgrading the analytical capabilities of flow analysis systems. A monolith column is a continuous polymer rod [117] with a single physical structure which exhibits a bimodal pore size: macropores and mesopores, with average diameters of ca. 2 μm and 13 nm, respectively. Monolithic columns can be made from different materials, e.g. metal oxides, graphite carbon, organic polymers or silica. The latter are more widely used and can also be functionalized [118,119]. Comparative studies between silica monolithic columns and 3.5 μm particle-based columns showed similar van Deemter curves, although much lower back pressures were observed with the former [120]. These two features made possible the straightforward coupling of these columns into flow analysis systems and opened a new trend in flow analysis. In this context, the commercialization of monolithic columns can be viewed as a milestone: firstly, in 1998, CIM – convective interaction media – disks were made available by BIA Separations (Ajdovščina, Slovenia) and later, in 2000, Merck K.G. (Darmstadt, Germany) also introduced this kind of stationary phase, branded as Chromolith [117]. Nowadays, monolithic columns can be purchased from many chromatographic column

manufactures. The current availability of monolithic columns in different sizes and with various stationary phase compositions explains the reduced use of lab-made columns in recent developments in flow analysis chromatographic methodologies.

In 2003 the first work combining flow analysis and monolithic columns was published [121]. The manifold relied on a commercial FIAlab 3000 system (FIAlab® Instruments, USA) featuring a Chromolith® Flash RP-18e (25 mm length × 4.6 mm i.d. column). The FIAlab 3000 system is designed to perform sequential injection analysis and includes a syringe unit and a peristaltic pump for propulsion of solutions, and a spectrophotometric detector, all fully integrated. Using this manifold, the chromatographic determination of salicylic acid and its ester methylsalicylate, in pharmaceutical preparations was achieved. This approach was named sequential injection chromatography (SIC), and thereafter several works followed under this designation as reviewed by Chocholouš et al. [122]. Currently a second generation of this approach is being explored making use of a more powerful equipment, SIChromII™ system (FIAlab Instruments, USA) [123].

Another approach developed by combining flow analysis and monolithic columns was multisyringe chromatography (MSC) [124]. MSC exploits the four-syringes set of the multisyringe equipment (likewise in SIC systems, syringe pumps are also used for solutions propulsion) to manage fluids during the implementation of chromatographic methodologies. A comparison between SIC and MSC approaches has been subject of discussion in scientific reviews [125–127].

SIC and MSC approaches have been termed medium pressure chromatography by several authors [128–132] because the syringe units of lab-on-valve and multisyringe systems can withstand back pressures of up to 25 bar. Due to this technical feature, the coupling of 10 cm length monolithic columns in these systems is common. The propulsion of solutions using syringe pumps offers as main advantages the high precision and long-term performance stability in dispensed volumes. The main disadvantages of these propulsion units are the need for a computer to control the syringe piston pace and the non-continuous flow, as the syringe barrel volume defines the maximum dispensed volume (ideally, each chromatographic run is limited by the volume of the barrel). Furthermore, performing gradient elution with syringes is not straightforward, and if miniaturization of these systems is intended, reducing the size of the syringe becomes a limitation.

In parallel with the developments of SIC and MSC, monolithic columns have also been tested in flow injection chromatographic systems (FICS). Following the analytical

system's pressure criteria, many authors [128,130,132,133] designated FICS as low-pressure chromatographic systems, based on the technical specifications of the solution propulsion devices, most commonly peristaltic pumps (which withstand a max. pressure of ca. 5 bar). The first study of this approach dates to 2004 by Connelly et al. who demonstrated the determination of inorganic ions in waters. These authors used a manifold comprising a peristaltic pump to propel the mobile phase, an injector, a 2.5-cm length monolithic column coated with a surfactant and a spectrophotometer [134]. Several works exploiting FIA systems and monolithic columns have been published since then, taking advantage of the well-established potentialities of flow injection systems along with the aforementioned compatibility of monolithic columns with the typical devices used in FIA manifolds, particularly, the liquid propulsion devices and injection valves. The advantages of conventional FICS, in contrast to their counterparts, include the simplicity of operation (the use of a computer is not mandatory), versatility in the manifold assembly, lower implementation cost, and better suitability for downsizing when portable systems are envisaged. Two reviews comparing SIC and FIC approaches are available, published in 2009 and 2013 [128,131].

To complement the current state-of-art, this chapter aims to provide a different insight into the low-pressure flow injection chromatography systems. By considering flow manifolds equipped with low-pressure solution propulsion devices and chromatographic columns, particular emphasis will be placed on manifold assembly with respect to the propulsion devices used, sample injection strategies, stationary phases and detection systems employed. Additional considerations regarding analytical system performance, as reported in published articles, are also discussed to enable readers to assess the ruggedness, feasibility and capabilities of these systems. To this end, research articles based on flow injection chromatography were gathered through a comprehensive search in the Scopus database. An iterative procedure was also carried out by evaluating the citations and references of each article to collect, to the best of our knowledge, all pertinent information for this review. It must also be added that an exhaustive compilation of flow analysis systems featuring lab-made columns was not the objective of this review. The reader is invited to proceed to the following pages in parallel with Figure 5 and Table 7 appended in the end of this chapter.

A 2.1. Manifold

A 2.1.1. Mobile Phase Propulsion Systems

The eluent propulsion units most frequently used in low-pressure chromatographic manifolds are peristaltic pumps. The reasoning for this choice is their affordability, ease of use (PC control is not mandatory), low maintenance and the continuous flow provided. Additional advantages include the wide range of commercially available peristaltic pump models, varying in size, number of rollers, tubing caliber and material, which can be selected to best suit specific needs.

In FICS, peristaltic pumps from Ismatec [135–137] or micro peristaltic pumps from BVT [138] were found, with the most frequently used models being Minipuls 2 or Minipuls 3 from Gilson. The latter, according to the manufacturer, can withstand a maximum back pressure of ca. 5 bar. As mentioned, the flow rate can be adjusted according to the tubing calibre and the rotation speed of the peristaltic pump (from 0.3 µL/min to 40 mL/min); tubing made of different materials is commercially available from various distributors and can be selected based on the chemical compatibility of the solutions.

Peristaltic pumps are more frequently used in FICS with monolithic columns of 0.5 or 1.0 cm in length. The maximum reported flow rate was 2.6 mL/min using a monolithic column 5.0 mm length x 4.6 mm i.d. [139]. Conversely, the longest chromatographic column coupled with peristaltic pumps consisted of a set of guard column plus the column (one Chromolith 10 mm × 4.6 mm i.d. plus one Chromolith Flash 25 mm × 4.6 mm i.d., both from Merck) [133,140] operating at a flow rate of 1.03 mL/min. In the latter conditions, a mobile phase containing up to 50 % (v/v) ACN was used, which is more favourable for reducing back pressure.

In the majority of the FICS featuring peristaltic pumps, Tygon® tubing was used, and no incompatibility was reported for mobile phases containing up to 70 % (v/v) methanol (MeOH) [141], or up to 20 % (v/v) ACN [137]. Additionally, the use of Tygon® MHLL tubing was reported - due to its superior chemical resistance to organic solvents - for carrier solutions containing up to 90 % (v/v) ACN [140]; Viton® tubing was also employed for methanol propulsion [142].

In low-pressure chromatographic manifolds, peristaltic pumps are commonly used to propel the mobile phase through a single channel under isocratic elution. Notwithstanding, peristaltic pumps featuring more than one channel are generally preferred, as they enable the simultaneous management of more than one solution.

Examples of the aforementioned include the use of two channels to deliver the same mobile phase, which are merged at a confluence point before the chromatographic column, thereby reducing the load exerted on each propulsion tube. This strategy was demonstrated by Connolly et al. [134] using a 25 mm length monolithic column. The use of multichannel peristaltic pumps combined with selection valves to manage different mobile phases during the chromatographic analysis was also a strategy followed. The combination of these devices enabled the switching of the mobile phase composition to a second one capable to more efficiently elute the most retained compounds [135,139,141,143–145]. This approach showed advantages in shortening the chromatographic run. Multichannel peristaltic pumps were also utilized to perform in-line post-column sample treatment in (i) amperometric determinations, where pH and ionic strength of the eluate were adjusted prior to detection to improve the electrochemical response [146–148]; or in (ii) chemiluminescence detection, where the derivatizing reagents were merged with the eluate before detection [139,145,149]. Aiming at reducing the human intervention, automatization of the column conditioning procedure was also referred taking advantage of multichannel peristaltic pumps and a selection valve [145].

Peristaltic pumps have also been used to perform gradient elution. Victory et al. [138] implemented a chromatographic system featuring two independently controllable micro-peristaltic pumps, each propelling a mobile phase solution containing 2 mM and 5 mM p-hydroxybenzoate, respectively. Gradient elution was achieved by gradually increasing the concentration of p-hydroxybenzoate in the mobile phase during the elution of inorganic anions.

A schematic representation of the abovementioned strategies with peristaltic pumps is presented in Figure 3.

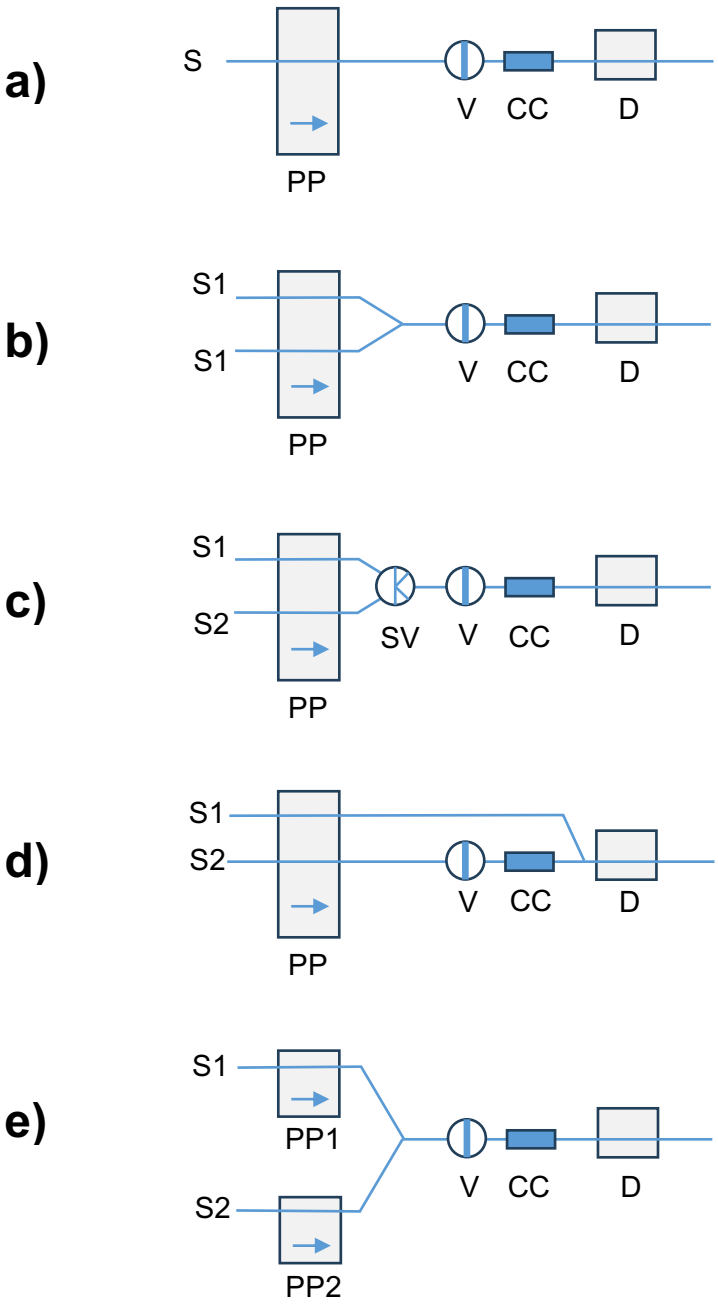


Figure 3 – Schematic representations depicting the use of single/multichannel peristaltic pumps in FICS: a) to perform single-channel isocratic elution; b) to reduce the peristaltic tubing effort toward column backpressure; c) combined with selection valves for non-isocratic elution; d) to perform post-column derivatization or eluate conditioning before detection; e) to perform gradient elution; S – solution; PP – peristaltic pump; SV – selection valve; V – sample injection valve; CC – chromatographic column; D – detector. Details in section A 2.1.1.

The second most used propulsion devices in FICS are milliGAT pumps commercialized by GlobalFia, Inc. These stepper motor-driven devices are computer controlled and enable precise and bidirectional propulsion of liquids. Two models are commercially available: LF (low flow) and HF (high flow), providing a flow rate range between 0.004 µL/s and 700 µL/s, and operating at low voltage (12–48 VDC). Furthermore, these devices combine the advantages of syringe pumps i.e. low pulsation flow and high precision volume delivery, with the advantages of peristaltic pumps, namely, continuous flow and no need for refilling. FIC manifolds reporting the use of milliGAT pumps [129,149–151] operated under a maximum pressure below 100 psi, with a pressure release valve maximum rated up to 100 psi typically coupled to the manifold. The maximum flow rate reported was 2.4 mL/min in a FICS featuring a monolithic column 5 mm in length x 4.6 mm i.d. [149]. The longest column used comprised a set of monolithic columns (guard column and main column) of 5 mm and 25 mm, respectively, operating at a flow rate of 1 mL/min [129]. In terms of solvent compatibility, mobile phases containing up to 25 % (v/v) ACN [129] and 36 % (v/v) MeOH were found reported [149]. MilliGAT pumps were used for both isocratic [150] and gradient [129,149,151] elution. In gradient elution operation mode, two pumps were operated synchronously, so that one accelerated its pace while the other deaccelerated.

Surprisingly, a single work was found using a gear pump for solutions propulsion [135]. Due to their intrinsic operating mechanism, these pumps provide an almost pulseless flow. Miniaturized model devices are commercially available and may be considered an alternative to overcome the tubing limitations associated with peristaltic pumps.

A 2.1.2. Sample Injection

The injection valve most frequently found in FICS is from Rheodyne, model 5041 [133,135,136,139,140]. With wettable parts of PTFE (Polytetrafluoroethylene) and polychlorotrifluoroethylene (Kel-F), and compatible with organic solvents, this injection valve can withstand pressures of up to 20 bar, is electrically operated, and offers versatility in loop volumes selection. These advantages make this valve one of the most expedite alternatives to perform sample volume-controlled injections in FICS with precision and reproducibility. The use of other electrically operated injection valves was also found, particularly models from Valco (models C12 [150,151] and SGE [129,152]). Manually operated valves (e.g. Rheodyne 5020), are also frequently used and present

the advantages of low cost and straightforward use, as they do not require external control.

A different strategy to perform sample injection in FICS was used by Batista et al. [137]. Using a two-way solenoid valve, the sample volume was defined by the flow rate and the actuation period of the solenoid, which alternately diverted between the carrier solution and the sample. These authors evaluated sample volumes ranging from 5 to 24 µL.

Loop volumes commonly used in FICS are within 10 and 50 µL interval. Nevertheless, in some works [135,139,143] larger sample volumes were explored seeking higher signal intensities. Garcia-Jimenez et al., in the determination of antioxidants, preservatives and sweetener additives in food and cosmetics, using a 5 mm-length monolith column, observed an increase in signal height with sample volumes up to 125 µL without compromising the analytes resolution [135]. Ballesta-Claver et al., using a similar column in the determination of phenolic compounds in health care products, were able to enhance the analytical signals with sample volumes up to 150 µL [139]. Tyrell et al. used a sample volume of 220 µL with CIM columns, coated with dipicolinic acid for the determination of copper [153]. Yalçin et al. were able to use loop volumes of 200 and 500 µL in the analysis of inorganic arsenic species using SPE cartridges [154]. Higher sample volumes, typically between 500 and 1200 µL, are used in analytical systems based on flow injection spectrophotometry. Due to the unique characteristics of this approach – which feature a spectrophotometric flow cell containing an adsorbent – it is explored the saturation limit of the adsorbent toward the analytes, aiming to improve the sensitivity of the analytical system [142,155–157].

Considering that higher sample volumes are less compatible with current trend in HPLC technique, that uses lower particle size (<5 µm) based columns, it is worth highlighting the advantageous higher overloading capacity of FICS in the development of analytical methods.

A 2.1.3. Chromatographic Columns

The chromatographic columns used in flow injection analysis systems can be divided into lab-made columns, commercial particle-based columns and commercial monolithic columns.

Concerns in the assembling of lab-made columns include the choice of supporting tube material for the stationary phase, the column's length and diameter, the composition

of the stationary phase, and the sealing strategy of the stationary phase at both column ends. It is worth noting that all said experimental details are often omitted, making it difficult to reproduce the published analytical procedures. Nonetheless, the following supporting tube materials were found reported, PTFE [158,159], acrylic [160], stainless steel [161] and glass [155]. To prevent loss of stationary phase at the column ends, the use of cotton wool [160] or glass wool [162] was found referred. Furthermore, details regarding the coupling procedure of lab-made columns are not clarified or schematized, being assumed that a leak free system is set resourcing to typical flow injection connectors (e.g. PTFE grippers, polypropylene or Tefzel® couplings, polypropylene end tube fittings). FICS featuring lab-made columns typically operate at flow rates of ca. 1 mL/min. The particle size of the stationary phase commonly ranges from 40 to 120 μm [155,157,158,160]. Alumina [115,158,159], C_{18} [144,161], and ion-exchange resin [160,163,164] are examples of stationary phases used. The dimensions of the lab-made columns are typically between 1 and 10 cm in length, and 1-4 mm in internal diameter. A more in-depth study of columns dimensions – covering both length (1–30 cm) and diameter (3–5 mm) – was conducted by Jiang et al., in the context of low-pressure ion chromatography [165]. Despite no discussion or correlation was provided between the columns' dimensions used and the chromatographic performance, chromatograms showing 8 to 10 eluting analytes are depicted. Still, as previously mentioned, a comprehensive review of lab-made columns in FICS is beyond the scope of this work.

An additional strategy in FICS involved the immobilization of the stationary phase inside a flow through cell, a technique referred to as flow injection solid-phase spectrophotometry. In this strategy, a typical procedure comprises placing a plug of wool at the cell's outlet and filling of the cell chamber with the stationary phase by means of a syringe [156,166]. Some works further combined this setup with an additional lab-made column placed in the flow manifold before the detector to improve the chromatographic separation [142,144,155]. A deeper compilation of works based on this approach was conducted by Matsuoka et al. [167].

The use of commercially available columns in flow analysis chromatographic systems has helped overcome many of the abovementioned practical issues inherent to the assembly and coupling of lab-made columns. Regarding commercial particle-based columns in FICS, three works were found. Vidigal et al. used a guard column Aminex HPX-87H (40 × 4.6 mm) from Bio-Rad, for the determination of sugars in wine samples, operating at a flow rate of 0.4 mL/min [168]; Costin et al. [152] used a C_{18} guard column from Waters for the determination of a mixture of amino acids at 0.8 mL/min. Batista et al [137] tested fused core columns commercialized by Supelco, with four different

stationary phases (C_{18}, pentafluorophenyl propyl, RP-amide and phenyl-hexyl; 5 mm x 4.6 mm, particle size 2.7 μm) operating at 0.6 mL/min in the separation of parabens in personal care products.

Regarding the use of disposable cartridges, Yalçin et al. illustrated the separation of As(III) and As(V) in a flow system featuring either anion exchange or reversed phase SPE columns (2 cm in length and 1.2 cm in diameter) operating at 4 mL/min [154]. No experimental details of the SPE column coupling to the FIA manifold were provided.

Monolithic columns are currently the most frequent choice in the development of a FICS due to the inherent features of this type of stationary phase as previously discussed (at the beginning of Chapter 2). Twenty works using monolithic columns were identified, which may be justified by their pioneering use in SIC systems and the resulting reliable chromatographic performance. Although various stationary phases are commercially available (i.e. phenyl-; cyano-; diol-; NH_2-; and Si-), C_{18} columns of 5 or 10 mm in length are most commonly used in the development of new methodologies. The maximum flow rates reported for monolithic columns in FICS were already referred in section 2.1.1. An additional relevant feature, compared to the previous lab-made columns, is the availability of commercial holders for monolithic columns with integrated male/female connectors. These holders enable efficient coupling of monolithic columns to the flow system minimizing dead volumes and the distances between column and injector, as well as between column and detector. This is an important practical aspect for reducing analyte dispersion in the mobile phase along the flow path [148,158]. Still within the context of monolithic columns, two works were found using CIM columns [136,153]: Tyrrell et al. illustrated the selective retention and elution of Cu(II) in environmental samples using a flow analysis system based on a single CIM column of 3 mm x 16 mm i.d. [153]; Garcia Jimenez et al. reported the resolution of sweeteners in beverages using a two disks set [136]. It is worth referring that short length CIM columns – after proper functionalization and coupled to HPLC systems – have shown analytical applicability in biological samples, e.g. in the separation of oligonucleotides [169,170], peptides [169], or membrane proteins [171,172], suggesting that adapting these applications to low-pressure manifolds seems foreseeable.

A schematic representation of the most usual abovementioned strategies is presented in Figure 4.

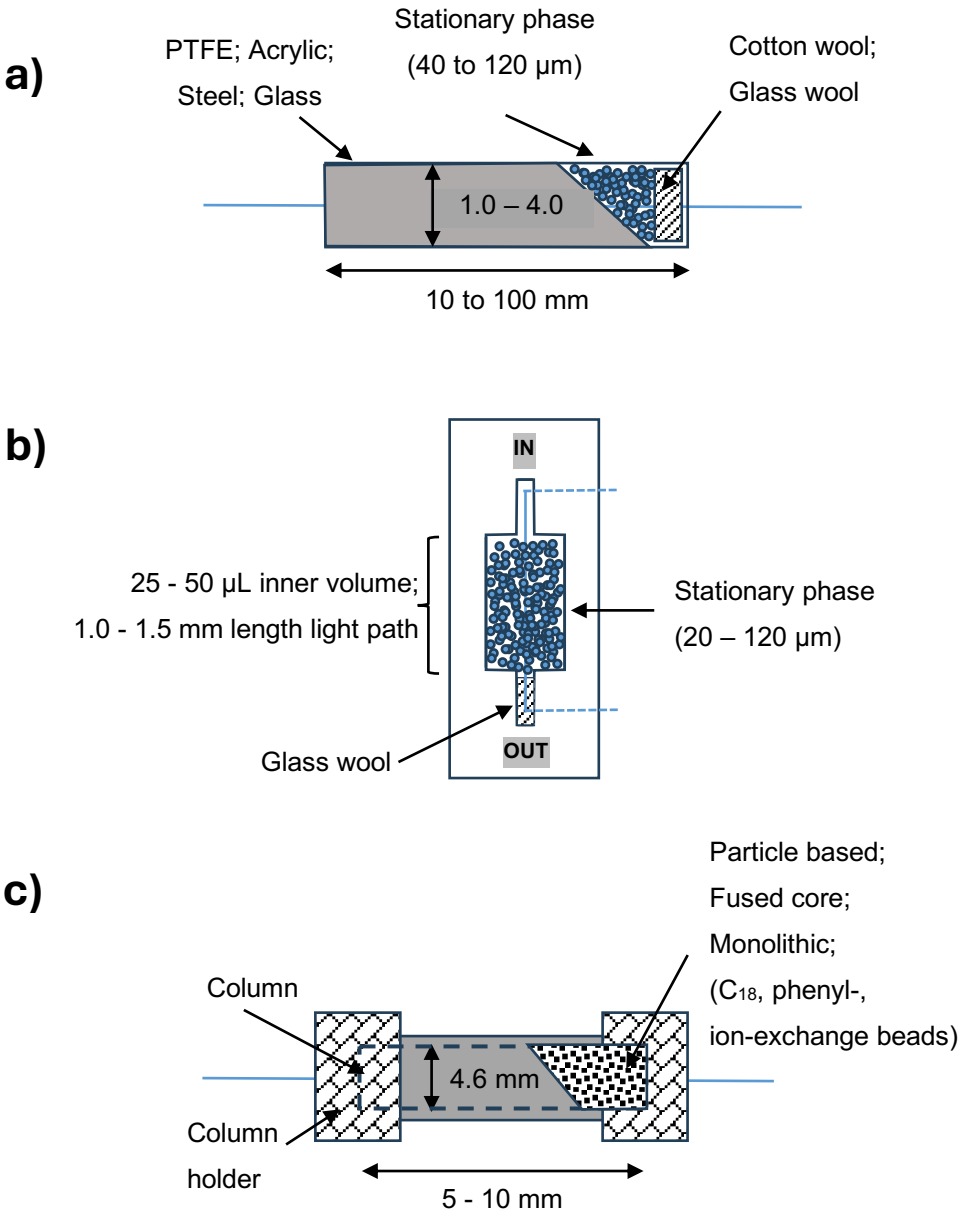


Figure 4 – Schematic representations of chromatographic columns used in FICS considering typical described characteristics. a) Lab-made chromatographic column (section view); b) spectrophotometric flow cell used in solid-phase spectrophotometry (frontal view); c) commercial chromatographic column (section view). Details in section A 2.2.3.

A 2.1.4. Detectors

Various types of detectors have been employed in low-pressure chromatographic flow systems. The less commonly used detection techniques include HGAAS (Hydride Generation Atomic Absorption Spectroscopy) [154], HGAFS (Hydride Generation Atomic Fluorescence Spectroscopy) [154] and ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry) [115,158]. In contrast, UV-Vis spectrophotometry is the most widely used. FICS with detection based on conductimetry, luminescence and amperometry have also been reported. In this scenario, some considerations will follow.

The coupling of UV–Vis spectrophotometers into FICS is straightforward. Cuvettes specifically designed for flow analysis, commercially available and featuring low internal volumes (typically between 8 and 18 μL), facilitate the use of conventional UV–Vis spectrophotometers in the development of analytical methodologies. The main concern in the system assembly is to reduce the distance between the column outlet and the detection point to minimize as much as possible the extra-column band broadening effect. In this regard, the use of spectrophotometers equipped with optical fibers allow for shorter distances between the column outlet and the spectrophotometric cell. Coupling of UV–Vis spectrophotometers in FICS was also exploited in the development of analytical methodologies based on solid-phase spectrophotometry as described in the previous section [142,144,155–157,162,166,167]. Although this approach simplified the system manifold, it has so far demonstrated limited separation capacity.

In published works involving luminescence detection, a post-column derivatization reaction prior to the detection stage is typically performed. To this end, a confluence and a reactor are incorporated into the flow network, positioned after the chromatographic column, to ensure proper mixing of the eluate with the derivatizing reagent. Ballesta Claver et al. [145] illustrated the feasibility of the chemiluminescent reaction of parabens with Ce(IV) in the presence of Rhodamine 6G. These authors used a confluence with three inlet channels, resulting in chromatographic peaks with widths of less than half minute. Similar flow networks were used in the determination of phenolic compounds in health care products [139] or in the determination of biogenic amines in urine [129]. The coupling of luminescence detectors to FICS manifold follows the same experimental considerations as those applied to UV-Vis spectrophotometers.

The use of amperometric detectors poses inherent cautions and the following three experimental aspects have to be addressed: (i) the working electrode should ideally be positioned in a wall-jet configuration relative to the eluate flow, to improve the analytical signal in terms of sensitivity, response time and effective cleansing of the

electrode surface; (ii) the accommodation of the electrodes should provide a short distance between the reference and working electrode to reduce the ohmic drop and baseline noise signal intensity; (iii) lastly, the use of amperometric detectors may require adjustment of the eluate composition, particularly its ionic strength and pH, which are critical to improve the signal-to-noise ratio. To fulfil all previous requirements, Santos et al., devised a confluence specifically conceived to be placed at the column outlet, to accommodate all electrodes and featuring additional inlet channels to enable the post-column adjustment of the eluate's pH and ionic strength [102].

Two works using conductometric detectors in low-pressure chromatographic systems are reported. Victory et al. adapted a conductivity cell from a commercial equipment for the determination of inorganic ions analysis after the eluate passed through a suppression cartridge [138]. Zhou et al. reported the determination of potassium in vitreous humour using a lab-made conductivity detector (no further details are provided) [173].

A 2.1.5. In-line Peripherals in FICS

The following paragraphs summarize the different approaches found to improve the ruggedness and reliability of FICS, tackling typical concerns of these systems, particularly, the need for mobile phase degassing, control of mobile phase flow patterns and the influence of temperature on analytical system performance.

Degassing of mobile phases is a standard procedure in HPLC analysis, with modern apparatus offering in-line deaeration via integrated vacuum pumps. In FICS, degassing the mobile phase to prevent air bubbles formation may not be mandatory. Two factors concur to this fact: (i) the typical compactness of FICS, which involve short tubing lengths and, mainly, (ii) the short length of the chromatographic column used which do not generate high back pressure as conventional HPLC columns do. In this scenario, some authors choose to degas the mobile phase, others do not mention this procedure and, lastly, others assume that degassing is unnecessary – which can be viewed as advantageous (one less experimental step) [174]. Nonetheless, two modifications in FICS were reported to address the issue of air bubbles formation: (i) Srisawang et al. [160] employed a three-way valve position before the column to divert air bubbles and; (ii) Silva et al. [102,175] reported the use of an end-line coil (a 3 m length PTFE tube with 0.8 mm i.d.) as back pressure regulator, which minimized air bubbles formation at the column outlet. This latter approach was also used by Chen et al. [176], who referred to it as decompression coil.

A common concern in FICS featuring peristaltic pumps, is the pulsed flow inherent to the operation mode of these solution propulsion devices – unlike the near-pulseless flow delivered by milliGAT pumps, syringe propulsion devices or conventional HPLC piston pumps. However, most published works on FICS do not emphasize this issue and references to the use of pulse dampeners are scarce: Hart et al. used a lab-made pulse suppressor mounted between the peristaltic pump and the injector [161]; Pelletier et al. used a commercial pulse dampers model Lo-Pulse 21 [177]. It is commonly referred that signal baseline pulsations are not so particularly significant as peak shapes at naked eye do not exhibit a stair-like pattern. This behaviour is often attributed to the inherent damping effect of the column within the FICS [134].

The effect of temperature increase was found in three works: (i) Lingyun Yu et al. [164] reported the determination of chloride, bromide and iodide ions by ion exchange chromatography and UV-Vis, based on the post-column discoloration reaction of indigo carmine in the presence of $KBrO_3$. This reaction is accelerated by halogen ions and by elevated temperature; (ii) Connolly et al. [134] reported a reduction of the system back pressure when heating of a 2.5 cm length monolithic column was performed; (iii) Victory et al. [138], also reported a reduction of the system back pressure along with a decrease in the chromatographic run time by 2 min, when the eluents reservoirs were heated to 45 °C. The latter case embodies a performance improvement of a chromatographic system relying on a 1 cm length column, achieved by heating the mobile phase.

Other in-line *peripherical*s reported in the literature include the use of nylon syringe filters (1 µm pore-size) as nominal filters placed in the eluent line between the peristaltic pump and the injector, aiming at extending the column's lifetime [148,175]; in LPC systems utilizing milliGAT pumps for propulsion of the mobile phase, a pressure release valve (model PRV-100; GlobalFIA) was placed between the milliGAT pump and the injection valve, to protect the milliGAT pumps in case of excessive back pressure [129,150,151]. Furthermore, the use of a cooling bath to mitigate the temperature increase of the carrier solutions by the milliGAT pumps functioning was also reported [129].

A 2.1.6. Manifold Control and Data Acquisition

Manifolds that do not require additional control equipment or dedicated software typically rely on manual injection valves and peristaltic pumps [146,148,168,175,178]. In contrast, manifolds incorporating solenoid valves for solution management and electromechanically actuated injection valves (e.g. for solutions composition selection)

require custom-built external control [135,141,145]. Specifically, manifolds using MilliGAT pumps for mobile phase propulsion were fully automatized using LabView software [129,149–151,179].

With respect to data acquisition, current FICS benefit from dedicated data processing software provided by commercially available detectors. This allows data acquisition and analysis to be accomplished in a much convenient and user-friendly manner. Furthermore, such software typically includes the basic tools for obtaining peak height and/or peak area data, which embodies the necessary data for quantification purposes.

Of notice, Garcia Jimenez et al. [136] referred the use of Statgraphics software package (ver. 5.0 Statistical Graphics Corporations, Englewood Cliffs, NJ, USA) for FIA peak and area analyses. The use of multivariate analysis has also been exploited to aid in the quantification of analytes not totally resolved in the FICS: Nussbaum et al. [140] used Partial Least Square analysis to perform the quantitation of Para Red, Sudan I and Sudan II dyes in food samples. Partial Least Square analysis was also used to the same referred purpose, in flow systems based on solid-phase spectrophotometry, where the kinetic behaviour in the retention – elution process of the analytes along the solid support was insufficient to separate the analytes [180,181].

This overall scenario concerning manifold control and data acquisition is well illustrative of FICS advantages in terms of the potential low implementation effort and ease of operation.

A 2.1.7. Size

One of the frequently cited advantages of FICS is their potential for miniaturization. However, despite the current availability of miniaturized detectors, particularly UV–Vis and electrochemical, most reported FICS still rely mainly on conventional bench detectors. A similar trend is observed with peristaltic pumps, where Minipuls models from Gilson are predominantly used despite the existence of commercially available miniaturized alternatives. The development of hand-sized and user-friendly chromatographic systems is still an unexploited area. Santos et al. presented photographs of FICS setups with amperometric [175] and UV-Vis detection [174] in the graphical abstracts of each respective work, from which it can be inferred that the total system length is less than 30 cm.

Victory et al. assembled a FICS to perform ion chromatography, comprising two micro-peristaltic pumps (1 cm × 1 cm x 6 cm) from BVT Technologies, a Rheodyne injection valve, a 1.0 cm × 0.4 cm reversed-phase monolithic column and a conductivity cell. The complete system (including the electronic pump control box) presented dimensions of ca. 10 x 10 × 10 cm, and a weight of 0.2 kg [138]. In 2011, Yu et al. [164], assembled a FICS measuring 25 × 20 × 20 cm for the determination of chloride, bromide and iodide in foodstuffs: the FICS consisted of a peristaltic pump, a lab-made ion-exchange column, an injection valve, an optical flow cell, an optical detector, and a homothermic heater. These authors also reported the manifold cost (ca. $USD 4000) emphasizing the low initial investment required if compared to conventional HPLC systems. Indeed, the lower cost of FICS, in terms of both implementation and operational expenses, is a widely acknowledged advantage over conventional HPLC apparatus [128,131,133–135,140,141,182].

A 2.2. Analytical Applications and Performance of FICS

A 2.2.1. Chromatographic Separation Mechanisms in FICS

The chromatographic separation mechanisms based on partition and ion-exchange have been predominantly exploited in FICS. Punctual illustrations of affinity chromatography and size exclusion chromatography were also found reported.

The higher number of analytical applications using partition mechanism in reversed phase mode is supported by the strong performance and broad applicability of stationary phases based on C_{18} chains bonded to the silica support. C_{18} columns used in FICS include particle-based columns[137,144,161] as well as numerous works employing monolithic commercial columns. Batista et al., tested commercial fused core particle columns (5 mm length x 4.6 mm i.d., particle size 2.7 µm) of, not only C_{18}, but also of pentafluorophenylpropyl, C_{16}-amide and phenyl-hexyl for parabens analysis in personal care products in reversed phase mode [137]. In this latter work, C_{16}-amide columns were deemed more suitable, although all tested stationary phases demonstrated separation performance. In a recent work, a monolithic column with a stationary phase of phenyl-propyl was used for the determination of volatile dicarbonyl compounds (these analytes were previously derivatized with o-phenylenediamine) in coffee brews [102]. C_{18} monolithic columns have also been employed in ion-pair chromatography where, a

surfactant is added to the mobile phase to pair with ionic compounds of opposite charge, thereby enabling their retention via partition chromatography. This strategy was applied in the determination of trigonelline in coffee extracts using perfluoroheptanoic acid [175] and 1-tetradecanosulfonate sodium [148] as ion-pair reagents. Additionally, tetrabutylammonium chloride was used in this context for controlled retention of As(III) and As(V) [154].

Ion-exchange chromatography in FICS was illustrated using both C_{18} columns and ion-exchange resins as stationary phases. In the former case, previous coating of the stationary phase is performed by means of a surfactant able to be strongly linked to the stationary phase: the use of didodecyldimethylammonium bromide, a double chained surfactant, was exploited for the selective retention of fluoride, chloride, nitrite, bromide and nitrate anions in water samples [138] as well as for niacin determination in coffee extracts [147]. Using the same coating agent, Pelletier et al. [177] illustrated the selective separation of iodate, chloride, nitrite, bromide and nitrate anions. In this context, is worth noting the use of carboxybetaine as a coating reagent used in a 1 cm length C_{18} columns, functioning as anion exchanger, which was exploited for chromatographic separation of nitrite, nitrate, iodide, and thiocyanate anions [183]. The use of ion-exchange resins as stationary phase in FICS include e.g. the use of diethylaminoethyl Sephadex A-50 ion exchange beads applied in hemoglobin typing from blood samples [160]. Ion exchange chromatography was also illustrated using activated alumina (in its acidic form) as the stationary phase for chromium speciation in water samples [115,159] and phosphorous ion analysis in steel matrices [158].

Affinity chromatography mode was demonstrated with a lab-made column by Narinesingh et al. [184] who quantified immunoglobulins in biologic fluids, using as affinity matrix protein A covalently immobilized to a 2-fluoro-1-methlpyridinium salt activated Fractogel.

Lastly, size exclusion chromatography was also reported with a low-pressure system by Strum et al. in the study of milk oligosaccharides using lab-made columns with graphitized carbon particles [185].

With respect to CIM columns, and following the same reasoning referred in section A 2.1.3, their use in FICS is foreseeable for separation mechanisms such as affinity, or hydrophobic interaction depending on their surface modification [186].

A 2.2.2. Performance of FICS

Low-pressure chromatographic systems based on lab-made columns were not extensively characterized in terms of chromatographic performance. Indeed, lab-made columns presented limited potential: typically, a reduced number of analytes is separated, and published case-studies focussed on samples of low matrix complexity, typically water samples or pharmaceutical formulations (cf. Table 7). An appealing application found using lab-made columns was the possibility to perform preconcentration of analytes, as demonstrated with metallic ions [115,158,187]. Approaches based on flow injection solid-phase spectrophotometry, have exploited the use of high sample volumes to enhance the figures of merit of the analytical response. Likewise, this strategy also found applications only in the separation of two to three analytes in samples of low matrix complexity [155,166,181]. An appealing application found using lab-made columns was the possibility to perform pre-concentration of analytes, as demonstrated with metallic ions [115,158,187]. Approaches based on flow injection solid-phase spectrophotometry, have exploited the use of high sample volumes to enhance the figures of merit of the analytical response. Likewise, this strategy also found applications only in the separation of two to three analytes in samples of low matrix complexity [155,166,181].

In contrast, research articles of FICS featuring commercial chromatographic columns commonly characterize the performance of the analytical system by reporting some figures of merit, namely peak asymmetry, resolution (Rs), the height equivalent to a theoretical plate (HETP), precision, organic solvent consumption and analysis rate (Table 7). From the data compiled, the following considerations may be drawn: the figures of merit are generally of the same order of magnitude, despite the distinct characteristics between the different manifolds such as the propulsion conditions used, the sample injection strategies, tubing i.d. (0.5 mm vs 0.8 mm) and the flow network configurations involved. HETP lies within 10 to 30 μm range, exception made in the work of Batista et al. (that used fused core columns, and 0.25 mm i.d. tube in the flow network), where HETP value reported was 10-fold less [137]. Asymmetry values are typically below 2, but values near 1 (gaussian shaped) were also reported [133,150,151]. Regarding this latter aspect, the connections between the column and the detector play a crucial role in minimizing post-column band broadening. The organic solvent consumption *per* chromatographic run is typically below 2 mL underscoring the environmentally friendly nature of these systems. The analysis rate throughput is another highlighted feature in FICS. Typically, more than 10 samples *per* hour can be analysed and, indeed,

chromatographic runs below 100 s for multi-analytes determination were illustrated [149,168].

An additional aspect in FICS performance is related to the long-term reproducibility of the manifold. Regarding maintenance routine in FICS, consulted works typically report only standard laboratorial practices aiming at extending the column lifetime (monolithic columns can generally be used during several months [102,148,168,174,178]). Considerations to enhance long term ruggedness of, particularly, peristaltic tubing were not found apart from the considerations already discussed in section A 2.1.1. There are no strict guidelines for when peristaltic tubing should be replaced, other than when signs of wear or degradation become evident. The lifespan of the tubing depends on factors such as the tightening of the tubing itself, the pump's rotation speed and the number of rollers of the peristaltic pump. Nonetheless, the available data concerning the within-day and day-to-day precision for analytical signals in some FICS is listed in Table 7. Typical relative standard deviations for these parameters are below 5 %.

A 2.2.3. Selectivity

The short length of chromatographic columns used in FICS is often considered a significant limitation in achieving the selectivity required by current analytical standards, especially when dealing with complex sample matrices. Nonetheless, analytical applications with biologic (e.g. urine [129], blood [160], vitreous humour [173]), environmental (e.g. saline water [153]) and food samples (e.g. brewed coffee [146,148,174], wine [168], soup [135,141,166]) have been carried out. In most of the analytical methodologies cited in this review, a validation procedure to assess each developed methodology was performed. Some examples are now given. Most commonly, the results obtained using the developed FICS were compared with those from HPLC reference procedures, and no significant differences were observed. This fact was illustrated not only in simple matrices e.g. in the determination of Fe(II) and Fe(III) in aqueous samples [163] or in the determination of parabens [143,145] and phenolics [139] in health care products, but also in more complex samples as food samples, e.g. in the determination of azo dyes in spices [140], methylxanthines in coffee brews [174] or sweeteners in beverages [136]. In the determination of six antioxidant compounds in coffee brews, the selectivity of the developed FICS method was further supported by mass spectrometry analysis [146]. In the determination of Cu(II) [153] in estuarine and coastal seawater samples, the selectivity of the implemented FICS was confirmed using certified reference materials. Likewise, in the determination of As(III)

and As(V) [154] in untreated, tap, and bottled water samples [154] and in the determination of Cr(III) and Cr(VI) in tannery wastewaters [176]. Also noticeable in some of the consulted works, the short sample pretreatment performed before analysis in the FICS. Some examples are now given, accounting that a validation of the developed methodology was made. In brewed coffees, no sample cleanup procedure was performed in the determination of methylxanthines [174] or chlorogenic acids [146]; likewise, for the analysis of sugars and ethanol in wine samples [168]. To determine azo dyes in spices [140], or parabens in cosmetics [151], a single solid-liquid extraction step was conducted.

A 2.3. Final Considerations and Future Perspectives

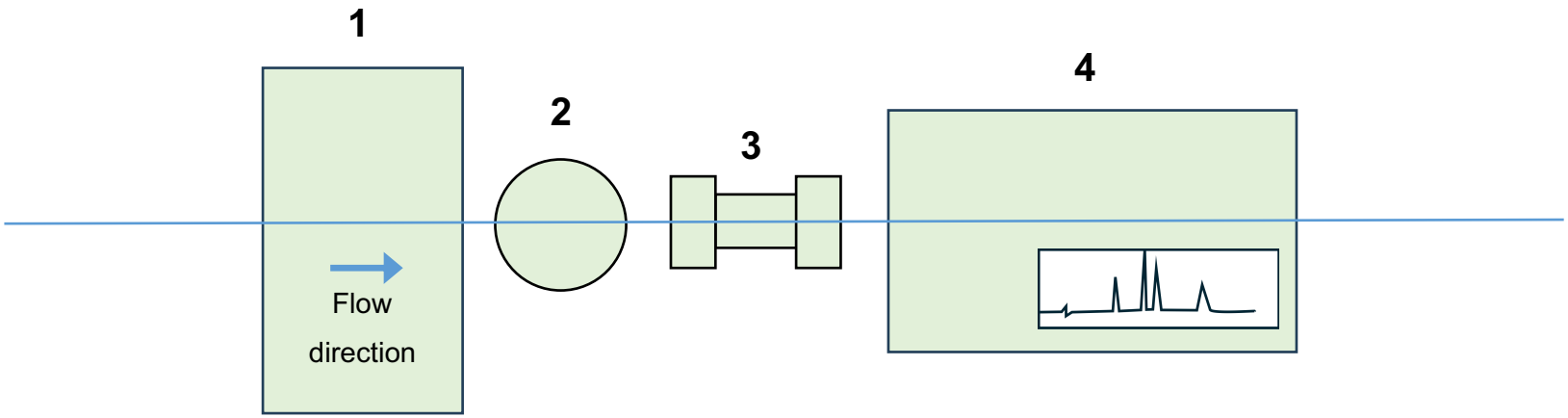
The following paragraphs will refer some last points-of-view concerning FICS state-of-art and future perspectives, in a brief and generalized way, considering three main aspects, (i) assembling and physical configuration, (ii) selectivity and (iii) applicability.

(i) From the current state-of-art, it resulted clear the simplicity, versatility and feasibility of FICS assembly, based on low-pressure solution propulsion devices, under varied flow network configurations, using different types of chromatographic columns (monolithic or particle packed bed), different types of detectors and often without the need for dedicated software control. The development of hand-sized systems of increased portability and low energy consumption is foreseeable, taking advantage of the currently available miniaturized propulsion devices for liquids, and the developments in e.g. miniaturized spectrophotometric or electrochemical detectors. Nevertheless, research on miniaturized FICS remains scarce and further analytical applications are needed to support this promising approach.

(ii) Despite of the columns' short length used in FICS, the selectivity of the chromatographic system can be fit to suit each case study through multiple strategies, some of which not easily achievable in conventional HPLC systems. Examples of the latter point include the straightforward configuration of the flow manifold, e.g. to modify the eluate's medium at the column outlet. Again, despite the column's short length, the selectivity can be adjusted by changing the temperature, by changing the mobile phase composition or by

modification of the stationary phase. One promising yet unexplored strategy in FICS context is the use of alternative monolithic stationary phases, such as diol, cyano, or amino, currently commercially available, which represent additional options for implementing *ad-hoc* FICS.

(iii) The features of FICS make it particularly suitable in process control tasks, whether at-line or in-situ, particularly in samples of near constant matrix composition (e.g. food samples, pharmaceutical formulations or biological samples), where the focus is on determining a limited number of analytes, preferably at micromolar concentrations or higher. The research works compiled in this review are well illustrative of this point. Indeed, additional examples, though not cited herein, could be drawn from studies exploiting medium or high-pressure pumps and short length chromatographic columns, which may be adapted for use with low-pressure propulsion devices. *Ad-hoc* FICS present low implementation costs, require low maintenance, and are easy to operate, making them an attractive alternative to conventional HPLC systems.



1 – Low-pressure propulsion device:

Most used devices: peristaltic pumps and milliGAT pumps. Depending on the flow network assembled, different elution strategies can be performed, i.e. isocratic or not isocratic (for details see section A 2.1.1). Typical flow rates used: 0.7 – 1.5 mL/min for 1 cm length columns.

2 – Sample injection:

Most used strategy: volume based, via rotary valve devices. Typical sample volumes: 10 to 50 µL. For sample injection using time based strategy see section A 2.1.2.

3 – Chromatographic column:

Current trend: use of commercial monolithic columns (typically of 5 and 10 mm length x 4 mm i.d.).
Most used chromatographic separation mechanisms: partition and ion exchange. Ion-pair, affinity and size exclusion strategies also exploited (see section A 2.1.3)

4 – Detection system:

Most used detection systems are UV-Vis spectrophotometers featuring flow through cuvettes with inner volumes typically of less than 50 µL. Luminescence and electrochemical techniques also fairly used (for further details see section A 2.1.4).

Figure 5 – Basic schematic of a FICS manifold: most used devices and strategies.

Table 7 – Summary of low-pressure flow injection chromatographic systems: manifold, case studies and chromatographic performance.

Year	Ref.	Carrier propulsion unit; Pump tubing i.d., mm; Elution mode; Flow rate, mL/min	Sample injection device; Sample volume, µL	Column; dimensions, mm (l x i.d.)	Detector	Manifold tubing i.d., mm	Observations	Nº of analytes	Analytes; Matrix	LOD£	Organic solvent consumption, mL/analysis	Analysis rate, h^{-1}	Peak asymmetry *	Rs*	HETP, µm*	Analytical signal precision (RSD) **
2024	[175]	Peristaltic pump (Gilson, Minipuls 3); Tygon, 1.02; Isocratic elution; 0.70	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 35	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6	Amperometry (Metrohm, µAutolab); Working electrode: boron doped diamond electrode (Windsor Scientific)	PTFE, 0.8	IP chromatography; Manifold not depicted; In-line filter; Back pressure coil; Post-column adjustment of the eluate; Manifold without PC-control.	4	Oxidizable and reducible compounds; Brewed coffee	10.3 µmol/L Caffeine	0.070 (ACN)	12	<3.24	2.27	30	< 5 % r
2024	[102]	Peristaltic pump (Gilson, Minipuls 3); Tygon, 1.02; Isocratic elution; 0.7	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 40	Monolithic, Phenyl-propyl, (Merck, Chromolith); 10 x 4.6	Amperometry (Metrohm, µAutolab); Working electrode: boron doped diamond electrode (Windsor Scientific)	PTFE, 0.8	RP chromatography; Manifold depicted; In-line filter; Back pressure coil; Post-column adjustment of the eluate; Manifold without PC-control.	4	α-dicarbonyl compounds; Brewed coffee	3.6 µmol/L Diacetyl	0.45 (ACN)	5	1.2	1.5	12.8	<3 % r; <7 % R;
2023	[149]	Stepper motor pump (Global FIA, MilliGAT LF); Gradient elution; 2.4	6-port, 2-position injection valve, (VICI Valco, C12), electric; 40	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	Chemiluminescence (assembling of the lab-made detector presented)	PTFE, 0.5	RP chromatography; Manifold depicted; Post-column adjustment of the eluate; Manifold PC-controlled.	4	Parabens; Cosmetics	0.05 µmol/L Methyl- and ethylparaben	3.8 (MeOH)	24	1.32	1.8	24	<3 % r;
2022	[140]	Peristaltic pump (Gilson, Minipuls 3); Tygon MHLL, 1.14; Isocratic elution; 1.03	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 10	Monolithic, C_{18}, (Merck, Chromolith); 25 x 4.6 + 5 x 4.6	UV-Vis (Agilent 8453); Flow through cell of 18 µL inner volume and 1 cm length optical path (Hellma 178–010-QS)	PTFE, 0.8	RP chromatography; Manifold depicted; Chemometric data treatment. Manifold PC-controlled.	3	Azo dyes; Food samples	nr	1.8 (ACN)	15	nr	nr	nr	<1 % r;

2021	[146]	Peristaltic pump (Gilson, Minipuls 3); Tygon, (nr); Isocratic elution; 0.7	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 34	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6	Amperometry (Metrohm, μAutolab); Working electrode: boron doped diamond electrode (Windsor Scientific)	PTFE, 0.8	RP chromatography; Manifold depicted; In-line filter; Post-column adjustment of the eluate; Manifold without PC-control.	6	Antioxidant compounds; Brewed coffee	6.73 μmol L^{-1} Caffeine	0.325 (ACN)	3	1.8	1.7	15	<1 % Rt; <1 % r
2018	[148]	Peristaltic pump (Gilson, Minipuls 3); Tygon, 1.02; Isocratic elution; 0.70	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 34	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6	Amperometry (Metrohm, μAutolab); Working electrode: boron doped diamond electrode (Windsor Scientific)	PTFE, 0.8	IP chromatography; In-line filter; Manifold not depicted; Post-column adjustment of the eluate; Manifold without PC-control.	2	Trigonelline; Brewed coffee	3.11 μmol L^{-1} Trigonelline	0.200 (ACN)	20	1.5	1.5	15	<3 % Rt; <5 % r;
2018	[133]	Peristaltic pump (Gilson Minipuls 3); Tygon MHLL, 1.14; Isocratic elution; 1.03	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 50	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6 + 25 x 4.6	Fluorescence (Jasco FP 6500); Flow through cell of 25 μL inner volume and 1 cm length optical path (Hellma 176.752-QS)	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled.	1	Glibenclamide; Beverages	0.1 mg L^{-1} Glibenclami-de	2.6 (ACN)	12	1.2	nr	nr	<1 % Rt; <1 % r; <2 % R
2016	[178]	Peristaltic pump (Gilson, Minipuls 3); Tygon, 0.76; Isocratic elution; 1.2	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 7.5	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6	UV-Vis (Ocean Optics HR4000); Flow through cell of 18 μL inner volume and 1 cm length optical path (Hellma 178.712QS)	PTFE, 0.8	RP chromatography; Manifold depicted. Manifold without PC-control.	2	Ofloxacin and ciprofloxacin; Aqueous samples inoculated with L. portucalensis strain F11	0.29 mg/L Ciprofloxacin	0.45 (ACN)	8	1.33	1.65	13	<2 % Rt; <10 % r
2016	[151]	Stepper motor pump (Global FIA, MilliGAT pump); Gradient elution; 2.4	6-port, 2-position injection valve, (VICI Valco, C12), electric; 45	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	UV-Vis (Hitachi, U-2000); Flow-through cell of 10 mm length of optical path (no further details)	PTFE, 0.5	RP chromatography; Manifold depicted; Manifold PC-controlled.	4	Parabens; Cosmetics	0.17 μmol/L Methyl-paraben	nr	nr (15 &)	1.0	1.7	59.5	<8 % R

2016	[150]	Stepper motor pump (Global FIA, MilliGAT LF pump); Isocratic elution; 1.5	6-port, 2-position injection valve, (VICI Valco, C12), electric; 45	Monolithic, C_{18}, (Merck, Chromolith); 10 x 4.6	UV-Vis (Lambda1, Perkin-Elmer); Flow through cell of 10 mm length of optical path (no further details)	PTFE, 0.5	RP chromatography; Manifold depicted; Manifold PC-controlled.	2	Parabens; Hygiene wipes	0.10 µmol/L Methyl-paraben	nr (0.75 ACN and 0.75 MeOH[&])	nr (12[&])	1.1	1.6	14.4	<2 % Rt; <6 % r; <9 % R
2015	[147]	Peristaltic pump (Gilson, Minipuls 3); Tygon, 0.89; Isocratic elution; 0.35	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 25	Monolithic, C_{18}, (Merck, Chromolith) coated with DDAB §; 10 x 4.6	Amperometry (Metrohm, µAutolab); Working electrode: boron doped diamond electrode (Windsor Scientific)	PTFE, 0.8	IE chromatography; Manifold depicted; Post-column adjustment of the eluate; Manifold without PC-control; Column stability > 1 month.	1	Niacin; Brewed coffee	0.79 µmol/L Niacin	0	10	1.7	0.8	128	<4 % Rt; <5 % r
2015	[163]	Peristaltic pump (Shanghai Huxi Analytical Instrument Plant); (nr; nr); Isocratic elution; (nr)	Six-way automatic injection valve (no further details);	Lab-made: cationic resin, 45–55 µm (lab-synthetized); 40 x 5	UV-Vis; Optical flow cell of 2.8 cm length optical path and 4 mm id (no further details).	PTFE, 0.5	IE chromatography; Manifold depicted; Post-column derivatization; Manifold PC-controlled.	2	Fe(II) and Fe(III); Waters	1.55 µg/L Fe(II)	0	2.5	nr	nr	nr	<2 % R
2014	[137]	Peristaltic pump (Ismatec, CP 78017-10), Tygon (nr); Isocratic elution; 0.6	Time-based sampling; Solenoid valves (no further details), electric; 10	Particle (fused core): phenyl-hexyl C_{18}, F5, and RP-amide, 2.7 µm (Supelco); 5.0 x 4.6	UV-Vis (Ocean Optics USB2000), Z-shaped flow-cell of 9 µL inner volume and 20 mm length optical path (FIAlab Instruments).	PEEK, 0.25	RP chromatography; Manifold depicted; Manifold PC-controlled.	3	Parabens; Personal care products	0.12 mg/L Methylpara-ben	1.4 (ACN)	7.5	1.28	1.72	1.02	<4 % r
2014	[168]	Peristaltic pump (Gilson, Minipuls 3); Tygon, (nr); Isocratic elution; 0.4	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 7.5	Particle: hydrogen form resin (BioRad, Aminex HPX-87H), 9 µm; 40 x 4.6	UV-Vis (Ocean Optics 2000), Flow-through cell of 18 µL inner volume (Hellma, 178.712QS)	PTFE, 0.8	Manifold depicted; Manifold without PC control; Minimum of 1000 injections.	2	Sugars and EtOH; Wines	2.3 g/L Fructose and glucose	0	36	nr	3.5	nr	<4 % r

2012	[174]	Peristaltic pump (Gilson, Minipuls 3); Tygon (nr); Isocratic elution; 0.85	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 11	Monolithic, C_{18}, (Merck, Chromolith) 10 x 4.6	UV-Vis (Ocean Optics HR4000), Flow through cell of 8 µL-inner volume and 1cm length optical path (Hellma, 178.713QS)	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold without PC control;	3	Methylxanthi-nes; Brewed coffee	2.0 µmol/L Theophylline	0.102 (ACN)	10	1.61	1.78	36.3	<3 % Rt; <5 % r
2012	[188]	Teledyne–Isco CombiFlash Rf 200 #, (nr; nr); Gradient elution; 5	variable (no further details)	Lab-made: graphitized carbon (Grace Davison), 60-90 µm; 120 x 28	Mass spectroscopy (Thermo Finnigan LCQ Duo quadrupole ion trap mass spectrometer)	nr	SE chromatography; Commercial chromatographic equipment.	n	Oligosacchari-des; Milk	nr	nr	nr	nr	nr	nr	nr
2011	[139]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 2.6	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 150	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	Chemiluminescence (Camspec CL-1)	PTFE, 0.8	RP chromatography; Manifold depicted; Two mobile phases managed by a PTFE rotary selection valve; Post-column adjustment of the eluate; Manifold PC-controlled.	5	Phenolic compounds; Health care products	0.023 µmol/L Salicylic acid	nr	12.8	nr	1.2	nr	<14 % r;
2011	[164]	Peristaltic pump (Shanghai Huxi Analytical Inst. Plant), (nr; nr); Isocratic elution; 0.7	Six-way automatic injection valve (no further details); 300	Lab-made: Anion-exchange resin, 30-35 µm (no further details); 130 ×	Optical detector and optical flow cell (no further details)	nr	IE chromatography; Manifold depicted; Manifold PC-controlled; Heating required for post-column derivatization reaction.	3	Cl^-, Br^- and I^- ions; Foodstuffs	0.002 mg/L Cl^-	0	4.2	nr	nr	nr	<3 % r;
2010	[143]	Peristaltic pump (Gilson, Minipuls 2); (nr; nr); Elution with two carriers; 2.6	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 50	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	UV-Vis (Hewlett Packard HP-8453); Flow glass cell of 1 mm length light path (Hellma 138-QS).	PTFE, 0.8	RP chromatography; Manifold not depicted; Two mobile phases managed by a Teflon rotary selection valve; Manifold PC-controlled.	4	Parabens; Cosmetics	12 µmol/L Ethylpara-ben and propylpara-ben	nr	20	nr	1.2	nr	<4 % r;

2009	[141]	Peristaltic pump (Gilson, Minipuls 2); Tygon (nr); Elution with two carriers; 2.4	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 50	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	UV-Vis (Hewlett Packard HP-8453); Flow glass cell 1 mm light path (Hellma 138-QS).	PTFE, 0.8	RP chromatography; Manifold not depicted; Two mobile phases managed by a Teflon rotary selection valve; Manifold PC-controlled.	3	Antioxidants; Foods and cosmetics	0.46 µg/mL Butylhydro-xyanisole	nr	22	nr	1.5	nr	<2 % Rt; <3 % r;
2009	[136]	Peristaltic pump Ismatec Reglo Digital, (nr; nr); Isocratic elution; 2.0	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 25	Monolithic, QA §§ (BIA Separations, CIM); 2 x (3 x 12 mm)	UV-Vis (Hewlett Packard 8453); Flow-through cell of 1 mm length light path (Hellma 178.710-QS).	PTFE, 0.8	IE chromatography; Manifold not depicted; Manifold PC-controlled.	3	Sweeteners; Foods and soft drinks	0.90 µg/L Saccharine	0	7	nr	0.8	nr	<2 % r
2009	[145]	Peristaltic pump (Gilson, Minipuls 2); (nr; nr); Elution with two carriers; 2.5	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 75	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	Chemiluminescence (Camspec CL-1)	PTFE, 0.8	RP chromatography; Manifold depicted; Two mobile phases managed by a Teflon rotary selection valve; Post-column adjustment of the eluate; Manifold PC-controlled.	4	Parabens; Cosmetics	0.019 µmol/L Methyl- and propylpara-ben	nr	20	nr	1.2	nr	<7 % r;
2007	[135]	Gear pump (Ismatec MCP-Z) ##; Elution with two carriers; 3.5	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 125	Monolithic, C_{18}, (Merck, Chromolith); 5.0 x 4.6	UV-Vis (Hewlett Packard 8453); Flow-through cell of 80 µL inner volume and 10 mm length light path, (Hellma 178.010-QS).	PTFE, 0.8	RP chromatography; Manifold not depicted; Two mobile phases managed by a Teflon rotary selection valve; Manifold PC-controlled.	8	Antioxidants, preservatives and sweetener additives; Foods and cosmetics	0.01 µg/mL Acesulfame K	nr	9	nr	1.1	nr	<3 % r;
2007	[129]	Stepper motor pump, (MilliGAT pump, Global FIA); Gradient elution; 1	6-port injection valve (Valvo, SGE), electric; 20	Monolithic, C_{18}, (Merck, Chromolith); 25 x 4.6 + 5 x 4.6	Chemiluminescence (PTFE reaction coil held against a photomultiplier tube (PMT; Model 9828SB: ETP)).	PTFE, 0.8	RP chromatography; Manifold depicted; Pressure release valve; Post-column derivatization; Manifold PC-controlled.	CS1: 6 CS2: 7	Alkaloids and biogenic amines; Urine	CS 1: 3×10^{-9} mol/L Morphine; CS 2: 2×10^{-8} mol/L Serotonin	nr	nr (CS1: 13 &; CS2: 7 &)	nr	nr	nr	<3 % r;

2007	[173]	HL-2 electronic pump (Model ZJ-3, Shanghai, China) ###; (nr; nr); Isocratic elution; 0.8	(no further details); 4	Lab-made: resin with exchange capacity of 0.04 mmol g^{-1} (no further details); 70 × 6	Conductivity (Lab-made)	nr	IE chromatography; No manifold depicted.	1	K^+ ion; Vitreous humour	1 mmol/L K^+ ion	nr	nr (6 &)	nr	1.5	nr	<3 % r; <5 % R
2006	[153]	Peristaltic pump Gilson, Minipuls 312), PVC (nr); Elution with two carriers; 1.43	6-port, 2-position injection valve, (Rheodyne, 7125); electric; 220	Monolithic, RP-SDVB) §§§, coated with dipicolinic acid (BIA Separations, CIM); 3 x 16	Chemiluminescence (polyethylene tubing fixed onto the front of a photomultiplier tube (PMT; Model H9319-02)).	PEEK, 0.127	IE chromatography; Manifold depicted; Manifold PC-controlled.	1	Copper ion; Environmental water	5 µg/L	0	7	nr	nr	nr	<4 % R
2006	[142]	Peristaltic pump (Gilson Minipuls 2); Viton (nr); Elution with two carriers; 1.6	6-port, 2-position injection valve, (Rheodyne, 5041); electric; 1200	Lab-made: C18 (Sep-Pak); 300 mg, i.d. 10 mm; Flow cell filled with Sephadex G-25, 20–80 µm	UV-Vis (Hewlett Packard 8453); Flow-through cell of 50 µL inner volume and 1 mm length light path (Hellma 138-QS).	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled. Two solid-phases; Solid-phase spectrophotometry.	2	Saccharin, aspartame; Sweets and drinks	0.3 µg/mL Saccharine	nr	10	nr	nr	nr	< 2 % r
2006	[177]	Sage Multi-Range syringe pump, model M365 (Thermo Electron Corporation, Beverly, MA, USA) ####; Isocratic elution; 1	Micro scale injection valve (Rheodyne, 7520); manually operated; 1	Monolithic, C_{18} coated with DDAB §; 5 x 4.6 and 10 x 4.6	Electrochemical detector Dionex ED-50A; Conductivity cell (1 µL)	PTFE, 1.6	Ion chromatography; Use of pulse dampers	CS1: 4; CS2: 5	CS1: Cl^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}; CS2: IO_3^-, Cl^-; NO_2^-; Br^-; NO_3^-	nr	nr	CS1: 15; CS2: 30	nr	nr	nr	nr
2004	[134]	Peristaltic pump (Gilson, Minipuls 2); (nr; nr); Isocratic elution; 0.2	(no further details); 25	Monolithic, C_{18} (Merck, Chromolith) coated with DDAB §; 25 x 4.6	UV-Vis (Hewlett Packard 1050) (no further details)	nr	IE chromatography; Manifold depicted;	5	Inorganic anions; Water	nr	0	nr (2 &)	nr	nr	nr	nr

2004	[138]	Peristaltic micro pump (BVT technologies); (nr), 1.016; Gradient elution; 0.33	Rheodyne injection valve (no further details); 25	Monolithic, C_{18} (Merck, Chromolith), coated with DDAB §; 10 x 4.6	Conductivity (cell adapted from a Dionex DX100 ion chromatograph)	nr	IE chromatography; Manifold not depicted; Heating of the carrier; Manifold PC-controlled.	6	Inorganic anions; Water	nr	0	3	nr	nr	11	nr
2004	[156]	Peristaltic pump (Gilson, Minipuls 2); (nr; nr); Isocratic elution; CS1: 1.10 CS2: 1.25	6-port, 2-position injection valve, (Rheodyne, 5041); electric; 1000	Flow through cell filled with C18-bonded silica (Waters, Millipore) 55-105 µm	UV-Vis (Hewlett Packard 8453); Flow-through cell of 50 µL inner volume and 1 mm length light path (Hellma 138-QS).	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled. Solid-phase spectrophotometry.	CS1: 2 CS2: 2	CS1: butylated hydroxytoluene (BHT) and n-propyl gallate (n-PG) CS2: BHT and butylated hydroxyanisole (BHA); Foods and cosmetics	CS1: 2.0 µg/mL nPG; CS2: 1.8 µg/mL BHA;	nr; (CS1: 3.7 (MeOH); CS2: 4.2 (MeOH)[&])	9	nr	CS1: 0.66 CS2: 0.77	nr	<2 % r
2003	[152]	Peristaltic pump (Gilson, Minipuls 3); PVC, 1; Isocratic elution; 0.8	6-port injection valve (Valco; SGE), (no further details); 30-70	Particle: guard column (C_{18}, Waters); (no further details)	Chemiluminescence (Lab-made)	PTFE, 0.5	RP chromatography; Manifold depicted; Post-column derivatization; Manifold PC-controlled.	CS1: 2 CS2: 2	Aminoacid mixtures	CS 1: 0.03 µmol/L Tryptophan; CS 2: 2 µmol/L Tryptophan	0	nr (CS 1: 20; CS 2: 30 [&])	nr	nr	nr	<4 % r
2003	[160]	Peristaltic pump (FIA-lab); Tygon, (nr); Gradient elution; 0.8	6-port injection valve (no further details); 80	Lab-made: DEAE §§§§ - Sephadex A-50 ion exchange beads, 40-120 µm (Phamasia Biotech); 20 x 3	UV-Vis (Spectronic Instrument, Spectronic 21); Flow through cell with 1 cm length optical path (Hellma, no further details).	PTFE, (nr)	IE chromatography; Manifold depicted; Three carrier solutions managed by a selection valve	3	Hemoglobin; Blood	nr	nr	< 1 (35 min *per* analysis)	nr	nr	nr	<5 % r

2003	[166]	Peristaltic pump (Gilson, Minipuls 2); (nr; nr); Isocratic elution; 1.4	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 1000	Flow through cell filled with C_{18}-bonded silica (Waters, Millipore) 55-105 µm;	UV-Vis (Hewlett Packard 8453); Flow-through cell of 50 µL inner volume and 1-mm length light path (Hellma 138-QS).	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled; Solid-phase spectrophotometry.	2	Butylated hydroxyanisole (BHA) and n-propyl gallate (nPG); Fatty foods and cosmetics	0.7 µg/mL BHA; 0.9 µg/mL nPA	2.8 (MeOH)	9	nr	0.928	nr	<2 % r
2003	[155]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 1.4	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 500	Lab-made: Sephadex SP C-25 ion-exchanger gel (Aldrich), 40-120 µm; 9 x 1; Flow cell filled with the same stationary phase	UV-Vis (Varian Cary 50); Flow through cell of 50 µL inner volume and 1 mm length optical path (Hellma 138-QS flow cell)	PTFE, 0.8	IE chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Solid-phase spectrophotometry.	2	Sulfamethoxa-zole and trimethoprim; Pharmaceutical preparations	0.6 µg/mL Trimetho-prim	nr	14	nr	nr	nr	1.40 % r
2002	[144]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 1.23	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 200	Lab-made; C_{18} bonded silica (Waters), 55-105 µm; 27 x 1; Flow cell filled with the same stationary phase	UV-Vis (Varian Cary 50); Flow through cell of 50 µL inner volume and 1 mm length optical path (Hellma 138-QS flow cell)	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Solid-phase spectrophotometry.	2	Paracetamol and caffeine; Pharmaceutical preparations	0.56 µg/mL Caffeine	nr	15	nr	nr	nr	<4 % r
2002	[162]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 1.14	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 600	Lab-made: C_{18} (Waters), 55–105 µm 40 x 2; Flow cell filled with the same stationary phase	Fluorescence (Perkin-Elmer LS-50 spectrofluorometer); Flow-through cell of 25 µL inner volume and light-path length of 1.5 mm (Hellma 176-QS)	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Solid-phase spectrophotometry.	2	Warfarin and Thiabendazole; Pesticides, pharmaceutical preparations, and waters	2.40 ng/mL Thiabenda-zole	nr	13	nr	nr	nr	< 1.5 % R

2001	[157]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 0.95	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 1000	Lab-made: Sephadex SP-C25 ion exchanger gel (Aldrich), 40 – 120 µm; 15 x 2; Flow cell filled with the same stationary phase	UV-Vis (Milton Roy 3000 array spectrophotometer); Flow through cell of 50 µL inner volume and 1 mm length optical path (Hellma 138-QS flow cell)	PTFE, 0.8	IE chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Solid-phase spectrophotometry.	2	Thiamine and pyridoxine; Pharmaceutical preparations	84 ng/mL Pyridoxine	nr	9	nr	nr	nr	≤ 3 % R
1999	[180]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 1.15	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 600	Flow through cell filled with C_{18}, 55 –105 µm;	UV-Vis (Milton Roy 3000 array spectrophotometer); Flow through cell of 50 µL inner volume and 1 mm length optical path (Hellma 138-QS flow cell)	PTFE, 0.5	RP chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Chemometric data treatment; Solid-phase spectrophotometry.	3	Paracetamol, caffeine and acetylsalicylic acid; Pharmaceutical preparations	nr	nr	nr	nr	nr	nr	nr
1999	[181]	Peristaltic pump (Gilson, Minipuls 3); (nr; nr); Elution with two carriers; 1.2	6-port, 2-position injection valve, (Rheodyne, 5041), electric; 600	Flow through cell filled with C_{18} bonded silica (Waters), 55 – 105 µm;	UV-Vis (Milton Roy Spectronic 3000 diode-array spectrophotometer); Flow through cell of 50 µL inner volume and 1 mm optical path length (Hellma, 138-QS)	PTFE, 0.8	RP chromatography; Manifold depicted; Manifold PC-controlled; One selection valve to manage two carriers. Chemometric data treatment; Solid-phase spectrophotometry	3	Caffeine, dimenhydrinate and acetaminophen; Pharmaceutical preparations	nr	nr	nr	nr	nr	nr	nr
1999	[165]	LDB-09 electronic pump (no further details); Isocratic elution; 1.2	nr; 20	Lab-made: different columns prepared (further details in reference)	Conductivity (Lab-made)	nr	IE chromatography; Experimental conditions for additional 8 case studies are presented.	7	Aminoacids; Organic acids; Metallic ions; Inorganic anions; Aqueous samples	nr	0	nr (3 [&])	nr	nr	nr	nr

1998	[154]	Peristaltic pump (Gilson, Minipuls 3), (nr; nr); Isocratic and "step-gradient elution"; 4	Rheodyne (no further details); 200 or 500	SPE cartridge, RP 55–105 µm; and anion exchange 37–55 µm (Waters, Millipore); 20 x 12	Hydride generation atomic absorption spectrometry (Varian, SpectrAA-5) and hydride generation atomic fluorescence spectrometry (PS Analytical, Excalibur 10.003)	nr	RP chromatography; Manifold depicted; *ca*. 100 sample analysis *per* cartridge unit; Two carriers; Manifold without PC control.	2	Inorganic arsenic species; Water	0.2 ng/mL As(III)	0	40	nr	nr	nr	nr
1995	[187]	Peristaltic pump (Alpkem RFA300); (nr; nr); 0.287	Time based sampling; Sampler (part of the Alpkem RFA300 equipment);	Lab-made; Capillary tube of 10 m x 0.5 mm and microcolumns of 2.5 x 2; (Further details in reference)	UV-Vis (part of the Alpkem RFA300 equipment)	nr	Manifold depicted; Pre-concentration procedure; Two carriers manually changed.	2	Pairs of metal ions: Cu(II) and Co(II), Co(II) and Fe(III), Fe(III) and Zn (II), Mn(II) and Pb(II); Water	0.2 nmol/L Co(II)	0	30-60	nr	1	nr	<2.2 % r
1991	[184]	Peristaltic pump (Gilson, Minipuls 2), (nr; nr); Isocratic elution; 0.7	nr; 100	Lab-made: Protein A immobilized into a Fractogel support (Bioprobe Int. Inc.); 1 cm^3	UV-Vis (ISCO U.V.); Flow through cell with 5 mm optical path (no further details)	PTFE, 0.5	Affinity chromatography; Manifold depicted; Three way switching valve to divert either the binding or eluting buffer streams over the affinity column; Manifold without PC control.	1	Immunoglobu-lin G; Serum and plasma samples	nr	0	6	nr	nr	nr	3 % r; <4 % R
1989	[161]	Peristaltic pump (Gilson, Minipuls 2), (nr; nr); Isocratic elution; 1.0	6-port, 2-position injection valve, (Rheodyne, 5020), manually operated; 100	Lab-made; C_{18} (Waters, Sep-Pak), 40 µm; 50 x 4	Amperometry (Metrohm 641 VA potentiostat and a Metrohm 656 electrochemical detector); GCE	PTFE, 0.5	RP chromatography; Manifold without PC control.	1	Vitamin A; Multi-vitamin preparation	300 pg Vitamin A	nr (3.20 (MeOH) [&])	nr (12 [&])	nr	nr	nr	<4 % r

1985	[115]	Peristaltic pump (Gilson, Minipuls) (nr; nr); Elution with two carriers; 1	Rotary injection valve (no further details)	Lab-made; activated alumina, acid form (no further details); 25 x 1.5	ICP spectrometer (Jarrell-Ash ICAP 9000).	nr	IE chromatography; Pre-concentration procedure; Two carriers manually changed; Manifold without PC control.	2	Cr(III) and Cr(VI); Water	0.2 µg/L Cr(VI)	0	nr (36 &)	nr	nr	nr	<3 % r
1985	[158]	Peristaltic pump (Gilson, Minipuls) (nr; nr); Elution with two carriers; 1	Rotary injection valve (no further details)	Lab-made; activated alumina, acidic form (BDH) 75-120 µm; 100 x 1.5	ICP spectrometer (Jarrell-Ash ICAP 9000).	nr	IE chromatography; Manifold depicted; Pre-concentration procedure; Two carriers manually changed; Manifold without PC control.	2	Phosphorus and iron; Water	0.6 µg/L Phosphorus	0	nr (36 &)	nr	nr	nr	<2 % r

- maximum pressure rated (mpr) 13 bar; ## - mpr 20 bar; ### - mpr 3 bar; #### - mpr 2.4 bar; § - DDAB – didodecyldimethylammonium bromide; §§ - QA - quaternary amine groups; §§§ - SDVB - styrene-divinylbenzene; §§§§ - DEAE - diethylaminoethyl; RP – reversed phase; IE – ion exchange; IP – ion pair; SE – size exclusion; £ - lowest limit of detection reported within the studied analytes; & - estimated; nr - not referred; CS - case study; * - lowest value; ** - RSD, relative standard deviation; r, repeatability; R, reproducibility and Rt, retention time

PART B

Low-Pressure Chromatographic Flow Systems with Amperometric Detection

Synopsis

Part B of this PhD thesis focused on studies performed using *ad-hoc* low-pressure chromatographic flow systems with electrochemical detection, for the determination of different analytes in coffee samples. These studies demonstrated that tailored flow systems, through proper selection of monolithic columns and electrochemical detection strategies, can perform rapid, low-cost, and selective determination of analytes in complex matrices.

Chapter B 1 presents the development of a flow injection chromatographic system using multiple pulse amperometry detection to expand the range of electroactive analytes that can be monitored in a single chromatographic run. The flow system features a 1 cm-length monolithic column and exploits multiple pulse amperometric detection for the parallel determination of oxidizable and reducible compounds in green coffee samples. The electrochemical detection approach relied on alternating anodic and cathodic potentials to selectively detect compounds with distinct redox behaviour during the elution process. This chapter presents the methodological studies, including the selection of the ion-pair reagent, its concentration, and the electrochemical detection parameters, followed by the results of the coffee brew analyses.

Chapter B 2 further expanded the applicability of low-pressure chromatographic systems by targeting volatile α-dicarbonyl compounds in coffee brews. These compounds play an important role in the formation of aroma and flavour attributes in coffee and are indicators of thermal reactions occurring during the roasting process. This case study presented different analytical challenges due to the high reactivity, volatility, and low concentration of the target analytes. This chapter addressed these challenges by integrating a fan-assisted extraction strategy with derivatization, before the chromatographic analysis. The chromatographic flow system relied on a 0.5 cm-length phenyl monolithic column.

The developed methodologies are aligned with green chemistry principles, due to reduced solvent consumption and, consequently, lower effluents generation. Furthermore, these case studies illustrated the potentialities of flow injection chromatographic systems to perform fast chromatographic runs at low cost, for multi-analyte determination in green and roasted coffee samples. These case studies reinforced the potential of flow injection chromatographic systems as practical analytical tools suitable for routine and resource-limited laboratory environments.

Chapter B 1 was adapted from the following paper:

***Silva, A. R**.; Almeida, P. J.; Santos, J. R. Multiple pulse amperometry in low pressure chromatography for parallel determination of oxidizable and reducible compounds. Analysis of a green coffee extract as a case study.*

Manuscript published in Talanta

DOI: 10.1016/j.talanta.2023.125016

Chapter B 2 was adapted from the following paper:

***Silva, A. R.**; Almeida, P. J.; Santos, J. R. Fan assisted extraction and low pressure chromatographic system with a phenyl monolithic column for amperometric determination of volatile α-dicarbonyl compounds in coffee brews.*

Manuscript published in Microchemical Journal

DOI: 10.1016/j.microc.2024.111689

Chapter B 1

Multiple Pulse Amperometry in Low-Pressure Chromatography for Parallel Determination of Oxidizable and Reducible Compounds. Analysis of a Green Coffee Extract as a Case Study

Abstract

In this chapter, the determination of both oxidizable and reducible compounds within a single chromatographic run is exploited for the first time, using a low-pressure chromatographic system and multiple pulse amperometric detection. The case study selected focussed on the analysis of green coffee extracts. The separation of the compounds was carried out using a 1-cm length monolithic column and an eluent prepared by mixing an aqueous solution of an ion-pair reagent, perfluoroheptanoic acid (PFHpA), and acetonitrile. The parallel determination of oxidizable and reducible compounds was performed by application of two consecutive pulses, E_1 = +1.6 V and E_2 = −1.5 V. At anodic conditions, the chromatographic peaks for 5-caffeoylquinic acid (5-CQA), caffeine and 5-feruloylquinic acid (5-FQA) were detected, while at cathodic conditions, a chromatographic peak was ascribed for trigonelline. In the developed methodology, the use of multiple pulse amperometry provided better sensitivity if compared to previously described amperometric methodologies determining either oxidizable or reducible compounds. Detection limits for the referred compounds of ca. 1 × 10−5 mol/L, an analysis rate of 12 h^{-1} and an acetonitrile consumption of 0.07 mL per analysis were achieved. The approach presented demonstrates the possibility of combining low pressure chromatographic systems and multiple pulse amperometry, in the development of new, low-cost and fast methodologies for multi-analyte determinations.

B 1.1. Introduction

The low-pressure chromatographic flow systems are an analytical approach that embody the coupling of short-length monolithic columns into traditional low-pressure flow analysis systems [134]. The immediate advantage resulting from this strategy is the selectivity increase in the referred flow analysis systems to a level not formerly achievable, provided by the chromatographic column and its separation capacity of sample's compounds. Supported by the inherent versatility of the flow systems, some different manifold configurations of low-pressure chromatographic systems have been reported, using distinct solution propelling devices and/or detection systems, as earlier referred [147,174]. Only more recently, the coupling of amperometric detectors in low-pressure chromatographic flow systems was exploited [146–148]. This approach showed to be of straightforward implementation and widened the possibility to further exploit, in low-pressure chromatographic systems, the advantages of electrochemical sensors (e.g. their low operational cost, good sensitivity, the different commercially available electrochemical detectors) and/or the different electrochemical measurements modes currently present in conventional electroanalytical equipments.

In this scenario, this work combines the use of multiple pulse amperometry (MPA) as an electrochemical detection mode with a low-pressure chromatographic system. In MPA, consecutive potential values are imposed in loop to the working electrode, for defined periods. This electrochemical technique has been mainly used to prevent fouling of the amperometric detectors: generally, two or more potential values are employed, one at which the current intensity of the faradaic process is measured and the other(s) to perform the electrode's surface cleansing/conditioning. The basics of this electrochemical technique and examples of analytical applications within flow systems have been subject of different reviews [189–191]: these report the application of different potential-time profiles to the working electrode, the use of MPA in electrodes or microelectrodes of different sensing materials, the use of different flow system configurations (capillary electrophoresis, liquid chromatography or flow analysis systems), focussing environmental, pharmaceutical or food sample case-studies. A deeper exploitation of this electrochemical technique to perform multi-analyte determinations has been recently presented using FIA [192–196] and BIA systems [197–201]: either increasing or decreasing potential values are imposed to the working electrode to progressively enable the oxidation or reduction of different compounds. For quantification purposes, the resulting stair-like electrochemical signal requires some mathematical data treatment. The use of MPA in chromatographic systems or in capillary electrophoresis for multi-analyte determinations is, naturally, more frequent as individual

signals are obtained for each analyte; surprisingly, the use of MPA to determine both oxidizable and reducible compounds, within a single analysis run, was not found described. The fact that most of the case studies found, targets a defined class of compounds (presenting the same redox behaviour) may justify this scenario.

To the best of our knowledge, this work is the first one that combines MPA in low-pressure chromatographic systems, aiming the determination of both oxidizable and reducible compounds within a single chromatographic run. This approach is used in the determination of three of the well-known relevant compounds occurring in green coffee extracts: trigonelline, caffeine and 5-caffeoylquinic acid (5-CQA). Using a bare boron doped diamond electrode, trigonelline presents a cathodic signal [148] whereas caffeine and 5-CQA present anodic signals [146]. In this work, the eluent composition and the MPA conditions are studied. The methodology selectivity is discussed and the figures of merit obtained are compared with previously reported methodologies.

B 1.2. Experimental

B 1.2.1. Reagents and Solutions

Ultrapure water (resistivity > 18.2 MΩ cm) was used to prepare all solutions. All reagents were used as purchased.

For the chromatographic analyses, the eluents were prepared by mixing aqueous solutions of the ion-pair reagent (IPR) perfluoroheptanoic acid (PFHpA) (Sigma-Aldrich, 342041, ≥ 97.0 %) with ACN (Fisher Chemical, ref. A/0627/17). Formic acid (ChemLab, ref. CL00.1305.1000, 99-100 %) and acetic acid (VWR, ref. 84.528.290, ≥ 98 %) were used to acidify the eluent when needed.

The ionic strength adjustment (ISA) solution was prepared by dilution of hydrochloric acid (HCl) 37 % w/w (Thermo Fisher Scientific, ref. H/1200/PB17) to the concentration of 1.0 mol/L.

Trigonelline hydrochloride (Sigma-Aldrich, T5509, ≥ 98 %), 5-CQA (Sigma-Aldrich, ref. C3878, ≥ 99 %) and caffeine (Sigma-Aldrich, ref. 200-362-1, ≥ 99 %) were used in the preparation of standard solutions. The stock solution of 5-CQA was prepared each 3 days. The stock solutions of trigonelline and caffeine were prepared weekly.

B 1.2.2. Low-Pressure Chromatographic System Assembling

A flow system, similar to the one used in a previous work [146] was assembled. Briefly, it comprised a peristaltic pump (Gilson Miniplus 3) to propel both the eluent (tygon tubing of 1.02 mm i.d. – Gilson, Inc., F117938) and the ISA solution (tygon tubing of 0.38 mm i.d. – Gilson, Inc., F117933), a manual 4-way injection valve (Rheodyne, ref. 5020) featuring a 35 µL loop, column holder (Merck, Chromolith, ref. 1.52256.0001) which housed the monolithic column of 1 cm-length (Merck, Chromolith C_{18}, ref. 1.51452.0001) and a lab-made poly(methyl methacrylate) confluence (Figure 6), used to accommodate the three electrodes systems.

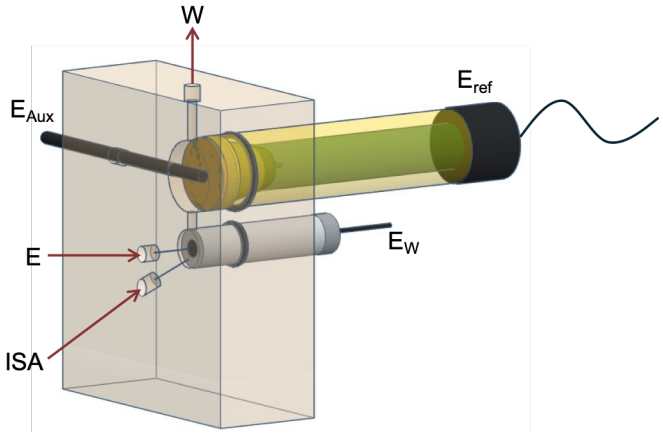


Figure 6 – Schematic representation of the lab-made confluence design, with two inlet channels (E and ISA). E - Eluent; ISA - Ionic strength adjustment solution; W - Waste; E_{Ref} - Reference electrode; E_w - Working electrode; E_{Aux} - Auxiliary electrode.

The electrochemical system was comprised of a boron-doped diamond electrode (Windsor Scientific, 3 mm Ø, ref. 3MMDIAM.BDD.PEEK), an Ag/AgCl reference electrode (Thermo, ref. 90-02, internal solution: 0.70 mol/L NaCl, ref. 90-00-02; external solution: 10 % w/w KNO_3, ref. 90-00-03) and a GCE as auxiliary electrode (Metrohm, ref. 6.1247.000). The electrodes were connected to a µAutolab (Metrohm, Ecochemie) controlled through software GPES v4.9. A schematic representation of the system used is presented in Figure 7 and the apparatus of the system used, as well as the monolithic column used is presented in Figure 8.

Tygon tubing of 0.38 mm i.d. (Gilson, Inc., F117933) and 1.02 mm i.d. (Gilson, Inc., F117938) was used in the peristaltic pump for propelling of the ISA and eluent solutions, respectively. For the remaining connections of the flow system, PTFE tubing 0.8 mm i.d. was used. A nylon syringe filter, 25 mm Ø, 1.0 µm (Whatman, 6750-2510), placed in the mobile-phase channel between the peristaltic pump and the injection valve, was used as

in-line filter. At the manifold exit, a 3 m length coil of PTFE tubing 0.8 mm i.d. was connected, to mimic a back pressure device and reduce air bubbles formation.

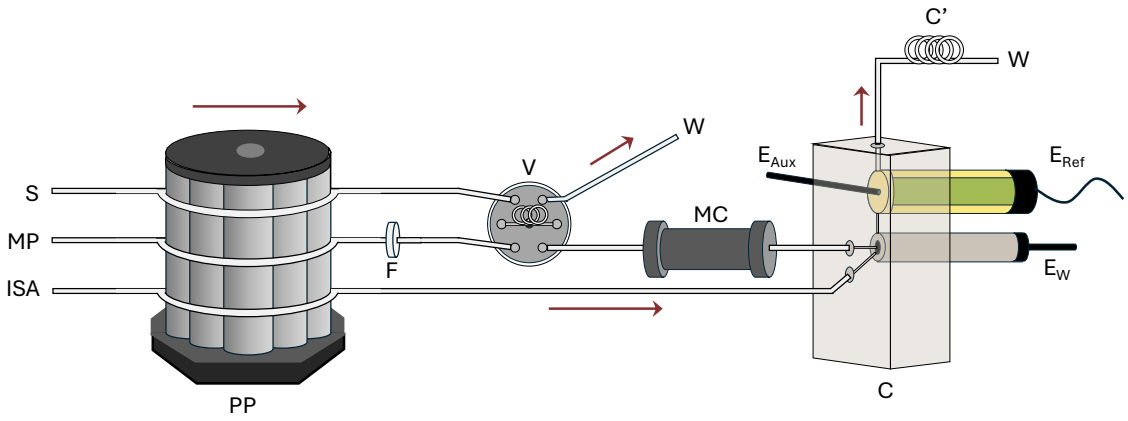


Figure 7 – Schematic representation of the low-pressure chromatographic system used. S – Sample; MP – Mobile phase; ISA – Ionic strength adjustment solution; PP – Peristaltic pump; F – Filter; V – Injection valve; MC – Monolithic column; C – Lab-made confluence; E_W – Working electrode; E_{Aux} – Auxiliary electrode; E_{Ref} – Reference electrode; C' – coil.

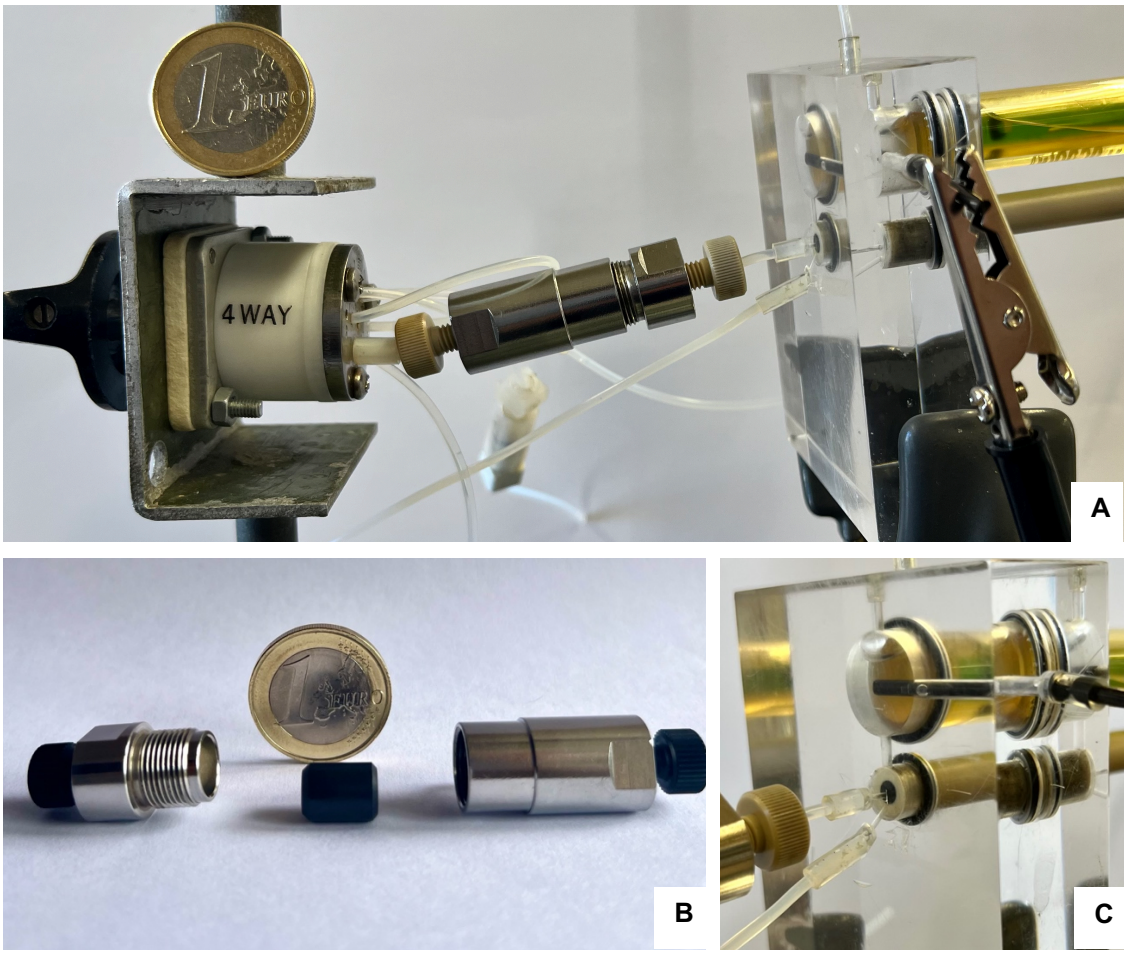


Figure 8 – Experimental apparatus of (A) the low-pressure chromatographic flow system with amperometric detection, and pH adjustment at the detection site; B – C_{18} monolithic column, with 1-cm length, and respective column holder; C – Lab-made confluence with the three electrodes system and two inlets (eluate and ISA solution).

B 1.2.3. Low-Pressure Chromatographic System Operation

The experimental conditions formerly used [146,148] were the ones initially selected in this work: the flow rate of the eluent was set to 0.70 mL/mL and the ISA solution composition was HCl 1.0 mol/L set at a flow rate of 0.14 mL/min. The estimated eluent dilution factor is 1.2 under these conditions. As previously referred [146], the low flow rate selected for the ISA solution enabled the adjustment of the eluent's pH and ionic strength at the detection site, improving the detection sensitivity without significantly diluting the eluate. At the beginning of each week or whenever a different eluent solution was tested, a conditioning period of ca. 60 minutes was necessary to achieve the column conditioning and for the electrochemical signal stabilization. Thereafter, the operation of the low-pressure chromatographic system followed the same experimental procedure previously described [148] likewise as typically performed in conventional flow injection analysis systems. At the moment of sample injection in the chromatographic system, the data acquisition was initiated under the electrochemical detection conditions selected.

The initial trials for oxidizable compounds were performed using direct current amperometry, by imposing a potential value of 1.6 V, the current intensity being measured every 0.1 s [146]. The initial trials for the reducible compounds were carried out using MPA, with E_1 = -1.5 V (0.03 s) for the analytes reduction and E_2 = +1.0 V (0.1 s) [148] for the electrode conditioning.

At the end of each workweek, the monolithic column was conditioned in ACN to increase the column life time. ISA and eluent solutions were previously deaerated in an ultrasonic bath before being used.

B 1.2.4. HPLC-DAD-MS/MS Analysis

For the mass spectrometry analyses, the 1 cm-length monolithic column was disconnected from the low-pressure chromatographic system and coupled to the HPLC-DAD-MS/MS system, so that a similar chromatographic separation can be obtained in both situations. The HPLC-DAD comprised a gradient pump, a DAD detector and an auto sampler (Finnigan Surveyor Plus, Thermo Electron Corporation); the mass spectrometry detector was a quadrupole ion-trap MS/MS spectrometer (Finnigan LCQ Deca XP Plus) equipped with an electrospray ionization source operating in positive ion mode. Remaining mass spectrometer conditions were: sheath gas (N_2) set at 40 (arbitrary units), auxiliary gas (N_2) set at 15 (arbitrary units), capillary temperature set at 325 °C, source voltage 5 kV and capillary voltage 15 V. The mass detection was

performed within 160–500 m/z mass range. For data acquisition and processing, software XCalibur v2.2 (Thermo Electron Corporation) was used.

B 1.2.5. Coffee Samples Preparation

Five green coffee samples (two of arabica var. and three of robusta var.) were analysed. Samples were milled and sieved (25 mesh) before the extraction procedure which was as follows: a rigorous mass of each sample was weighed (ca. 0.030 g) and transferred to a beaker with 20 mL of eluent. The mixture was left under gentle agitation for 10 min at 80 °C [202] and left to cool until room temperature. Lastly, the solutions were weighed and filtered, first with Whatman no. 1 paper and afterwards with a cellulose syringe filter 0.45 μm (Sartorius, ref. 17.762). This sample preparation procedure was performed before the analyses through either the low-pressure chromatographic flow system or the HPLC-DAD-MS/MS system.

Moisture of the green coffee samples was determined according to the reference procedure ISO (6673:2003). Moisture data were used to determine the samples dry weight.

B 1.2.6. Strategy Followed in the Analytical Method Development

The selected case study is based on two previous works that focussed the analysis of brewed green coffee, also using a low-pressure chromatographic system and a boron doped diamond electrode as amperometric detector: one for the determination of trigonelline [148] that is based on ion-pair chromatography prior to amperometric detection at cathodic potential; and the other for the determination of antioxidant compounds [146] that uses conventional reversed phase chromatography prior to the amperometric determination at anodic potential. The need for using different potential values, justify the approach herein exploited, i.e. the use of MPA to perform parallel determination of both oxidizable and reducible compounds within a single chromatographic run.

All studies performed involved the analysis of, both green robusta coffee brews and model solutions (containing trigonelline, caffeine and 5-CQA, being the latter two compounds representative of the most prevalent compounds determined under anodic conditions).

The followed strategy in the analytical methodology development encompassed the following three main stages: 1) evaluation of the eluent composition using previously described electrochemical conditions for trigonelline [148], and 5-CQA and caffeine [146]; 2) evaluation of the electrochemical conditions based on MPA to enable the determination of both oxidizable and reducible compounds within a single chromatographic run and; 3) evaluation of the developed methodology selectivity using the selected MPA conditions.

B 1.3. Results and Discussion

B 1.3.1. Eluent Composition Study

Preliminary studies aimed to select an IPR to control the elution of the compounds studied, particularly, trigonelline, without significantly interfere in the electrochemical and MS analysis (technique further used in selectivity evaluation studies – section B 1.3.3.). An option was made by PFHpA, a volatile IPR, formerly used in the determination of amino acids by MS/MS detection [203,204]. Cyclic voltammetry studies of model solutions of trigonelline, caffeine and 5-CQA, in the presence of PFHpA (5.0 mmol/L), showed no interference of this IPR in the electrochemical signals of the studied analytes (Figure 9), thereby fulfilling two of the abovementioned requisites.

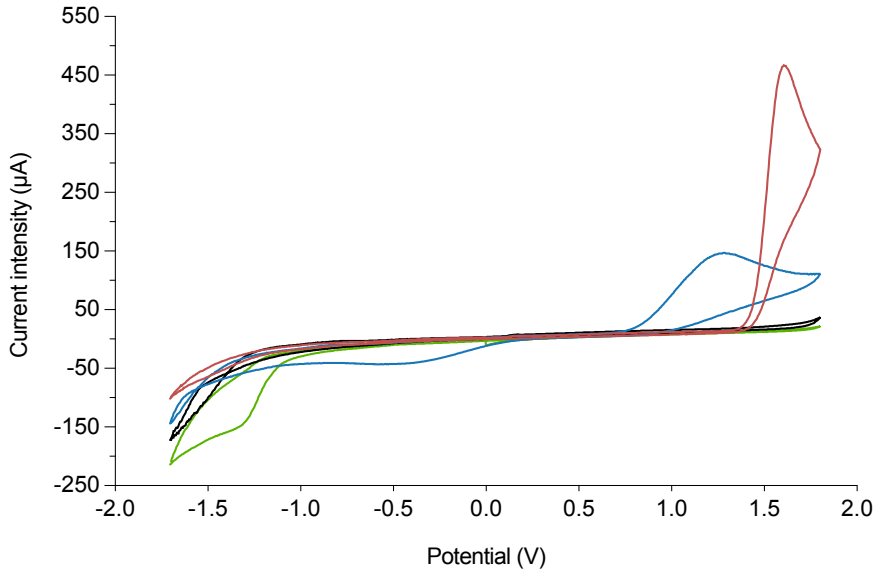


Figure 9 – Cyclic voltammograms for standard solutions of (—) caffeine, (—) 5-CQA and (—) trigonelline, 5.0 mmol/L. (—) Supporting electrolyte: PFHpA 5 mmol/L, pH = 2.0 adjusted with HCOOH. Electrochemical conditions: Scan speed: 0.5 V/s; $E_{initial}$ = 0.0 V; $E_{1st\ vertex}$ = + 1.6 V; $E_{standby}$ = 0.0 V.

Twelve different eluent compositions were initially tested using different values of PFHpA concentration, ACN content and pH value (Eluents 1 to 12 - Table 8). To track the elution order of trigonelline, 5-CQA and caffeine during the study of the eluents' composition, coffee samples, spiked coffee samples, and model solutions of the analytes were injected. The IPR was studied at the following concentration values: 0.5, 1.0 and 2.5 mmol/L. More concentrated solutions of the IPR are likely to significantly affect MS/MS detection and less concentrated solutions imply longer conditioning periods of the monolithic column and less interaction of the IPR with analytes [148]; ACN content was studied at 2 % and 5 % v/v, i.e. within the range usually employed in low-pressure chromatographic systems [147,148,174]. Two pH values were studied: 2.3 and 2.8. Due to the pKa value of trigonelline (2.78), higher pH values were not considered; additionally, since the lower operational pH value for monolithic columns is 2, lower pH values were also not studied. For eluents containing lower IPR concentrations, formic acid was chosen to adjust the desired pH level, due to its compatibility in MS/MS analysis. The results obtained in terms of retention time, asymmetry and resolution are presented in Table 8.

Table 8 – Influence of the eluent composition in the chromatographic parameters' retention time, resolution and asymmetry.

Eluent n°	[IPR] / mmol/L	[ACN] / % v/v	pH	Retention time*/ s			Resolution** (lowest value)	Asymmetry** (highest value)
				P1 (trigonelline)	P2 (5-CQA)	P3 (Caffeine)		
1	0.50	2	2.3	52.3	78.8	150.7	1.41	3.34
2	1.0	2	2.3	72.7	53.8	121.2	0.89	2.61
3	2.5	2	2.3	98.7	38.7	99.7	1.33	3.84
4	0.50	2	2.8	45.0	161.1	226.7	0.80	2.74
5	1.0	2	2.8	51.6	108.2	171.2	1.86	4.00
6	2.5	2	2.8	120.8	65.1	167.7	1.35	4.19
7	0.50	5	2.3	33.0	46.2	69.6	0.98	3.45
8	1.0	5	2.3	41.5	33.8	55.4	<0.80	3.15
9	2.5	5	2.3	63.2	25.7	49.4	1.49	3.40
10	0.50	5	2.8	32.3	64.2	113.2	<0.80	4.37
11	1.0	5	2.8	38.6	53.4	72.7	1.19	3.71
12	2.5	5	2.8	52.6	44.4	64.1	0.96	2.63
13	1.0	2	2.8	37.6	73.5	137.4	2.27	3.24

* - for peaks P1, P2 and P3, see Figure 10;

** - calculated according to [205].

One could observe in the chromatographic analyses of brew extracts, peaks presenting the same retention time and elution profile as trigonelline, caffeine and 5-CQA

peaks of model solutions, for all different eluent compositions evaluated. The chromatographic selectivity changed with different ACN contents, PFHpA concentrations, and pH value of the mobile phase. The highest ACN content tested caused an overlap of the chromatographic peaks due to a general decrease of the retention times regardless of the ion-pair reagent concentration or pH value used. The use of PFHpA enabled to control trigonelline elution thereby fulfilling the last requisite demanded in the selection of the ion-pair reagent. Indeed, in the experimental conditions tested, a change in the elution order of the peaks sequence could be observed i.e. with increasing PFHpA concentrations, trigonelline showed increasing retention times while the peaks potentially identified with caffeine and 5-CQA showed decreasing retention times.

The pH effect revealed to be also critical. A pH value lower than the pKa of trigonelline keep this compound with a single positive charge, favouring its interaction with the IPR; however, a too ionic eluent also shortened the retention time of 5-CQA and caffeine, reducing the resolution for these two peaks.

At this point, the best compromise between the separation of chromatographic peaks and the retention of trigonelline was achieved using a concentration of 1.0 mmol/L of PFHpA, ACN at a percentage of 2.0 % v/v and a pH value of 2.8 attained by adding formic acid to a final concentration of 0.1 % v/v. A final attempt to enhance the performance of the chromatographic elution was carried out by adjusting the eluent pH with acetic acid 2.0 % v/v, instead of formic acid 0.1 % v/v (eluent 13 – Table 8). This study was conducted based on previous expertise where a better chromatographic performance was achieved under reversed phase conditions [146].

The best results were indeed obtained using acetic acid. Using this acid content, the chromatographic run time analysis decreased while the chromatographic peaks remained well separated (Figure 10). In fact, comparing the chromatographic parameters obtained with the ones referred in previous works [146,148], the theoretical plate height values were of the same magnitude (within $3x10^{-5}$ to $5x10^{-5}$ m), but higher values of asymmetry were observed. It was reported that ion-pair chromatography exhibits better chromatographic performance than reversed-phase based elution [205], nevertheless that was not the case regarding the asymmetry parameter and the nature of the IPR used (or other variable) may be playing a more preponderant role. Further studies on this subject are outside the scope of this work and were not conducted.

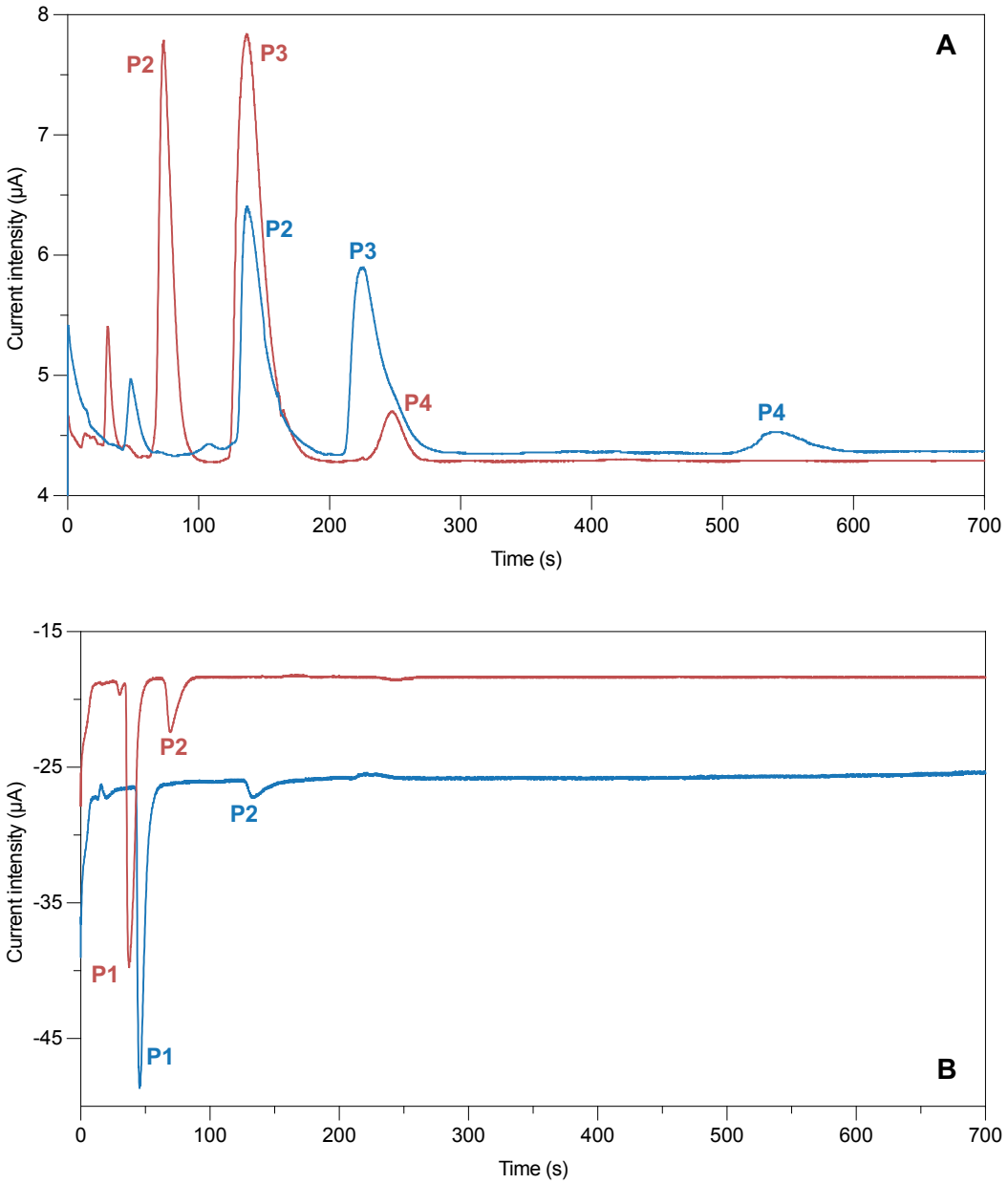


Figure 10 – Chromatograms of a coffee extract, spiked with (P1) trigonelline, (P2) 5-CQA and (P3) caffeine, obtained using an eluent containing PFHpA 1.0 mmol/L, ACN 2.0 % v/v and: (—) formic acid 0.1 % (v/v); caffeine, (—) acetic acid 2.0 % (v/v). Detection conditions: (**A**) anodic detection: +1.6 V (0.1 s); (**B**) cathodic detection: E_1 = -1.5 V (0.03 s), E_2 = +1.0 V (0.1 s).

The resolution values for the chromatographic peaks regarding trigonelline, 5-CQA and caffeine were higher than 1.5, indicating that the final experimental conditions selected for quantification of these compounds in green coffee extracts resulted in a quantitative separation of the chromatographic peaks.

For further research, an eluent containing PFHpA 1.0 mmol/L, ACN 2.0 % v/v and acetic acid 2.0 % v/v, was used.

B 1.3.2. Implementation and Evaluation of MPA for Detection in Parallel of Oxidizable and Reducible Compounds

Previous works that used a similar analytical manifold herein used, showed that caffeine and 5-CQA can be detected through direct current amperometry setting the following potential value and respective period: +1.6 V (0.1 s); trigonelline was referred to be detected setting the following MPA conditions: E_1 = -1.5 V (0.03 s) for reduction of trigonelline and E_2 = +1.0 V (0.1 s) for conditioning of the working electrode. Based on these data, for the parallel determination of these 3 compounds, MPA was tested using two pulses: E_1 = +1.6 V for 5-CQA and caffeine oxidation and; E_2 = -1.5 V for trigonelline reduction. For both potential values, equal periods were tested, namely 0.03 (the shortest period allowed by GPES), 0.10 and 0.15 s. The results obtained for a model solution containing trigonelline, 5-CQA and caffeine are depicted in Figure 11.

The results using the shortest period (0.03 s) revealed the analytical peaks with the highest intensity, more noticeable for trigonelline and 5-CQA peaks. In general, higher values of current intensities are sampled within shorter interval periods, which agrees with these results. Furthermore, one noticed an additional small oxidation signal at the retention time of trigonelline, and two reduction signals at the retention times of 5-CQA and caffeine, respectively. The reduction signal for 5-CQA is expected based on the cyclic voltammetry results, showing a reduction signal at -0.5 V, respectively. The oxidation signal for trigonelline and the reduction signal for caffeine, not initially previewed based on the CV results, may be due to the high potential change at the working electrode (ΔE = 3.1 V each 30 ms), which may be disclosing a (partial) reversible redox reaction for these compounds. Further studies are however necessary for a deeper understanding of these signals. In addition, these results showed that the selected potential value E_2 served therefore two purposes: to oxidize caffeine and 5-CQA compounds, but also conditioning of the working electrode, that is necessary to obtain a stable signal for trigonelline reduction as previously reported [148]. For the shortest period tested, a steady performance of the detection system, in terms of baseline noise (< 0.5 μA), drift (< 1.0 μA/100 s) and peak repeatability (CV < 5 %, n = 5) was observed. Given these results, a pulse duration of 0.03 s was chosen for further studies.

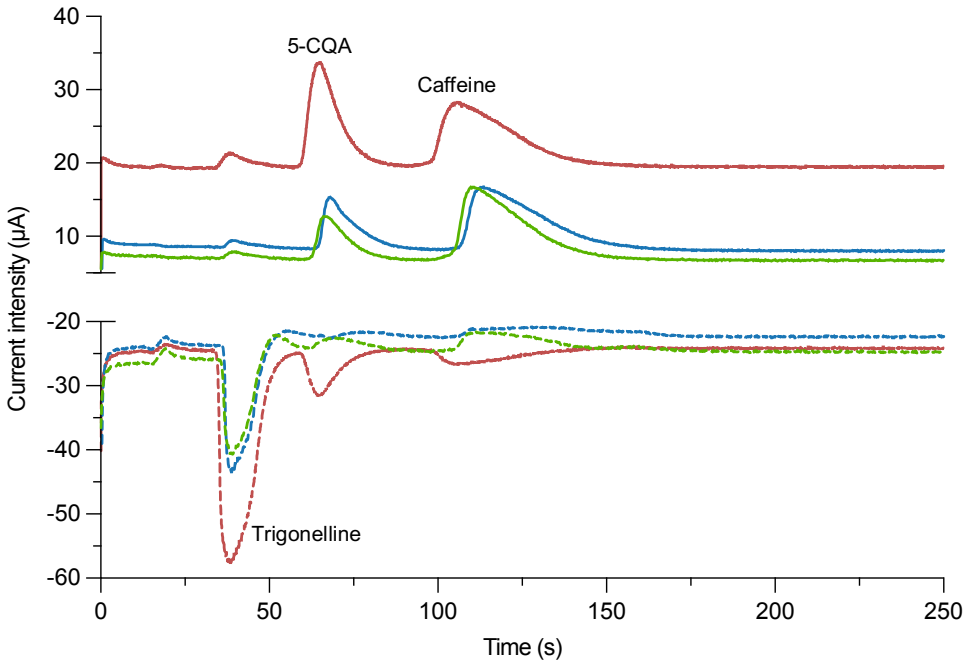


Figure 11 – Chromatograms of standard solution of trigonelline, caffeine and 5-CQA, 4.0×10^{-4} mol/L. Electrochemical conditions: MPA E_1 = +1.6 V (full line) and E_2 = -1.5 V (dashed line) for different pulse periods of both potentials: (—) 0.03 s; (—) 0.10 s and (—) 0.15 s.

B 1.3.3. Selectivity Evaluation

Based on the previous selected conditions of chromatographic elution and electrochemical detection, a typical chromatogram for a coffee brew extract is presented in Figure 12.

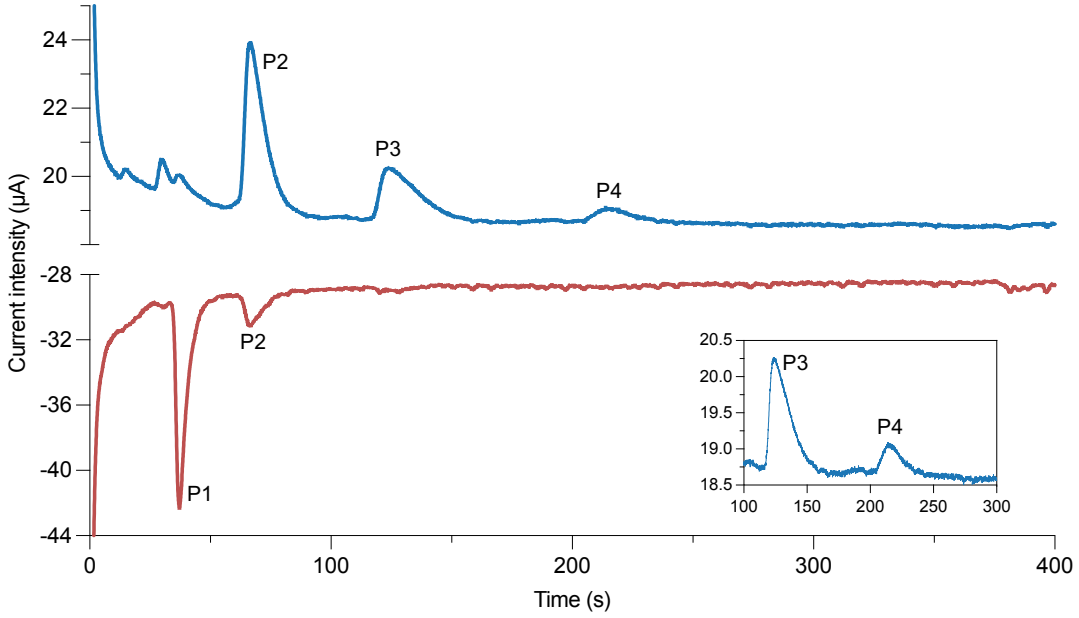


Figure 12 – Typical chromatogram obtained at the selected conditions of elution and detection for the parallel determination of oxidizable and reducible compounds in a coffee brew extract.

The results achieved so far with model solutions of trigonelline, 5-CQA and caffeine, with green coffee extracts and with green coffee extracts spiked with the referred analytes, have shown that three chromatographic peaks of the extracts eluted at the same retention time than trigonelline, 5-CQA and caffeine. The chromatographic profile of the coffee extracts is also similar to the one observed in a previous work [146], where caffeine and CGAs were determined using a similar low-pressure chromatographic system, although with an eluent composition based on a different ion-pair reagent. In addition to the previous considerations, two further studies were performed to ascertain the selectivity of the methodology developed: 1) the analysis of a green coffee extract using a HPLC-DAD-MS/MS and; 2) the analysis of a green coffee extract together with model solutions of trigonelline, caffeine and 5-CQA, using the low-pressure chromatographic system, at lower anodic and cathodic potential values.

In the analysis through HPLC-DAD-MS/MS, the system assembly was as previously referred (section B.1.2.4.). The eluent used was composed of 1.0 mmol/L PFHpA, 2 % v/v ACN and 0.1 % v/v formic acid (pH = 2.8), due to the MS detector limitation on the maximum acid content present in the eluent (1 % v/v). The HPLC-DAD-MS/MS chromatograms obtained were analogous to the ones observed using the low-pressure chromatographic system with amperometric detection. The results regarding the maximum wavelength and the highest mass fragments obtained are presented in Table 9 together with the respective compounds identification.

Table 9 – Mass fragments and corresponding compounds identified after HPLC-DAD-MS/MS analysis of a green coffee extract.

Peak	λ/nm	Precursor ion	Product ions	Compound	Standard/ Reference
P1	264	138	-	Trigonelline	Standard
P2	324; 243	355	191 (100), 179 (5)	5-CQA	Standard
P3	272	195	138	Caffeine	Standard
P4	324; 241	369	191(100), 193(10)	5-FQA	[206]

HPLC-DAD-MS/MS results of mass fragments and wavelengths of maximum absorbance supported the identity of trigonelline, 5-CQA and caffeine at retention times of peaks P1, P2 and P3, respectively. Additionally, it was shown that at the mentioned retention times, these compounds' fragments were the most prevalent. The last peak (P4) was identified as 5-feruloylquinic acid (5-FQA).

The electrochemical behaviour study of the coffee extract signals obtained, using lower anodic and cathodic potential values was conducted as follows. A green coffee

extract was prepared and analysed using the herein studied experimental conditions. Thereafter, a model solution containing trigonelline, caffeine and 5-CQA was prepared so that each respective chromatographic peak intensity was similar to the one observed in the green coffee extract. Consecutive injections of the coffee extract and model solution were performed, being the working electrode set to the following potential values: +1.6 V, +1.5 V, +1.4 V, +1.2 V, +1.0 V, +0.8 V and +0.6 V to study the electrochemical behaviour of the oxidizable compounds and; -1.5 V, -1.4 V, -1.3 V, -1.2 V and -1.1 V to study the electrochemical behaviour of the reducible compounds (Figure 13).

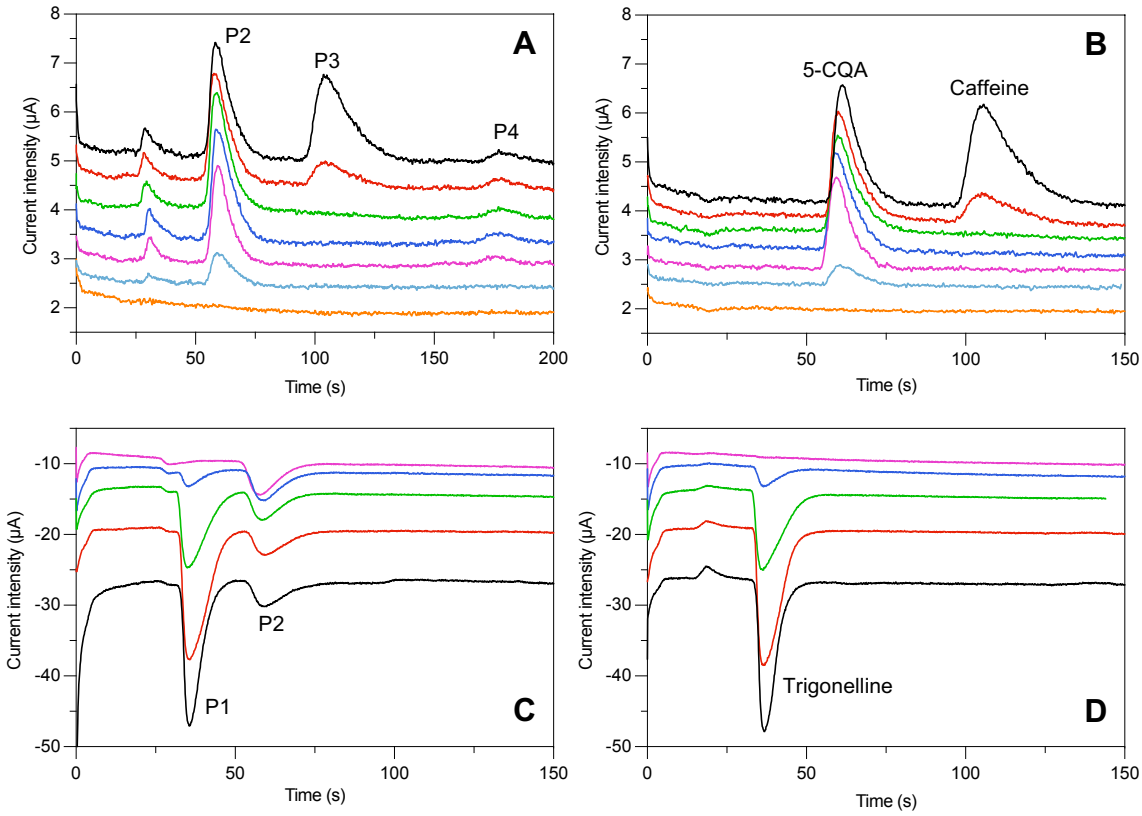


Figure 13 – Chromatograms obtained at different anodic potentials applied to the working electrode: — +1.6 V; — +1.5 V; — +1.4 V; — +1.2 V; — +1.0 V; — +0.8 V; — +0.6 V, for (**A**) Robusta coffee extract solution and (**B**) equimolar solution of 5-CQA and caffeine, 2.0 x 10^{-4} mol/L. Chromatograms obtained at different cathodic potentials to the working electrode: — -1.5 V; — -1.4 V; — -1.3 V; — -1.2 V; — -1.1 V, for (**C**) Robusta coffee extract solution and (**D**) standard solution of trigonelline, 1.0x10^{-4} mol/L.

The results showed a similar decrease of the chromatographic peaks intensity for trigonelline, 5-CQA and caffeine in model solutions comparing to the chromatographic peaks in the sample extracts presenting the same retention time as the referred compounds. This behaviour indicates that no additional compound with significant electrochemical activity co-eluted using the selected experimental conditions. Furthermore, the electrochemical behaviour and the MS/MS results for P4 were also in

accordance with previous discussion [146] supporting the identification of this compound as 5-feruloylquinic acid.

In sum, all evaluations carried out to ascertain the method selectivity were consistent that trigonelline, 5-CQA and caffeine can be determined in green coffee extracts, without significant interference of the coffee matrix under the selected experimental conditions.

B 1.3.4. Figures of Merit

Calibration curves for trigonelline, caffeine and 5-CQA using the abovementioned electrochemical conditions for parallel determination of both oxidizable and reducible compounds were performed and compared with the ones retrieved through reported individual anodic and cathodic measurements (Table 10). The interval of concentrations assayed (within $5.0x10^{-5}$ to $1.0x10^{-3}$ mol/L) was based on typical concentrations in coffee samples and the sample preparation procedure followed.

Table 10 – Calibration curve parameters for trigonelline, 5-CQA and caffeine determined using the low-pressure chromatographic flow system and different electrochemical conditions.

Calibration curve parameters	Trigonelline		5-CQA		Caffeine	
	MPA[a]	MPA (this work)	DCA[b]	MPA (this work)	DCA[b]	MPA (this work)
Slope/ $A.mol^{-1}.L$	0.73±0.08	0.88±0.10	0.08±0.01	0.33±0.03	0.22±0.01	0.47±0.03
Intercept/ µA	12.8±13.5	8.3±16.5	-0.04±1.46	-3.2±4.4	0.20±1.73	4.4±5.5
Correlation coefficient	0.9982	0.9981	0.9983	0.9991	0.9997	0.9993
Upper limit of linear response	$2.5x10^{-4c}$	$2.5x10^{-4c}$	$1.0x10^{-3}$	$1.0x10^{-3}$	$1.0x10^{-3}$	$1.0x10^{-3}$
Detection limit/ mol/L	$1.78x10^{-5}$	$1.76x10^{-5}$	$1.68x10^{-5}$	$1.18x10^{-5}$	$7.46x10^{-6}$	$1.03x10^{-5}$
Quantification limit/ mol/L	$5.92x10^{-5}$	$5.87x10^{-5}$	$5.59x10^{-5}$	$3.92x10^{-5}$	$2.48x10^{-5}$	$3.45x10^{-5}$

a - MPA as referred in [148]

b - direct current amperometry as referred in [146]

c – a second linear calibration curve can be established within the concentration range from 3.0×10^{-4} to 1.0×10^{-3} mol/L ($r = 0.9989$)

Using the herein selected MPA conditions, an increase of the baseline current intensity of ca. 5 µA and 15 µA (during measurements of the cathodic and anodic current intensity, respectively) was observed if compared to DCA conditions. This behaviour was expected due to the wide potential value change (3.1 V), at each 0.03 s, imposed to the working electrode. A further comparison of both methodologies showed similar figures of merit in terms of detection limit and linear response range, and a higher slope value for all analytes, especially for 5-CQA and caffeine compounds. This latter observation is

mainly due to the interval time parameter associated with the applied oxidation potential. While in the methodology based in DCA, sampling time was of 100 ms, in this work a sampling time of 30 ms was selected, and in general, for shorter interval times, higher faradaic currents are measured. The slight increase of the slope for trigonelline, may be correlated with the different conditioning potential used +1.6 V instead of 1.0 V as previously used [148].

In the overall, the herein presented methodology showed a better analytical performance, supported by the higher sensitivity and the capability of determining a higher number of analytes within a single chromatographic run. The analysis rate was of 12 samples/h, and the ACN waste was of 0.070 mL/sample.

B 1.3.5. Sample Analysis

Five green coffee samples were pre-treated and analysed using the developed methodology. The content values of trigonelline, 5-CQA and caffeine, were calculated by the external calibration method and the results are shown in Table 11.

Table 11 – Content values of trigonelline, 5-CQA and caffeine found in green coffee samples [a].

Sample	Trigonelline		5-CQA		Caffeine	
	Concentration	RSD [b]	Concentration	RSD [b]	Concentration	RSD[b]
Arabica Brasil	1.4 ± 0.1	0.15	4.1 ± 0.1	3.14	1.09 ± 0.04	3.63
Arabica Honduras	1.07 ± 0.01	1.11	3.7 ± 0.1	2.80	1.02 ± 0.04	4.34
Robusta Indonesia	0.81 ± 0.03	4.15	4.1 ± 0.1	2.75	1.55 ± 0.02	1.01
Robusta Uganda	1.03 ± 0.03	3.31	5.0 ± 0.2	3.53	1.87 ± 0.03	1.39
Robusta Vietname	0.94 ± 0.01	1.10	4.5 ± 0.1	2.93	2.07 ± 0.04	1.79

a – Values expressed as “g of analyte/g of dry weight bases x 100”.

b – Relative standard deviation (n=3).

The concentration values obtained for the referred analytes are within the typical content ranges occurring in green coffee samples as reported in the literature [54,207]. The chromatographic profile was similar in all samples analysed. CV values were lower than 5 % attesting the repeatability of the experimental methodology. These results support the suitability of the developed methodology to determine trigonelline, 5-CQA and caffeine in current green coffee samples.

B 1.4. Conclusions

This work is, to the best of our knowledge, the first one that combine low-pressure chromatography with MPA detection for the parallel determination of oxidizable and reducible compounds within a single chromatographic run.

In the case study selected, the determination of trigonelline and antioxidant compounds in green coffee extracts was achieved. The eluent composition was studied and PFHpA was used as IPR to aid in the retention control of the analytes. In this respect, it was also noticeable the impact of the eluent's composition upon the chromatographic selectivity control, even though a 1-cm length column was used. These results highlight the versatility of these columns in the development of *ad-hoc* analytical systems. The use of MPA increased the sensitivity (comparing to previously described amperometric methodologies) in spite of the wide interval of potentials applied and consequent capacitive currents generated. Trigonelline, 5-CQA, caffeine and 5-feruloylquinic acid compounds were identified and separately eluted. Calibration curves were established for trigonelline (under cathodic conditions), 5-CQA and caffeine (under anodic conditions).

This approach represents a new possibility of merging the known advantages of low-pressure chromatographic systems with the ones of electrochemical techniques. It has a great potential to tackle analytical chemistry challenges and/or improve analytical methodologies based on, likewise, chromatography and electrochemical detection.

Chapter B 2

Fan Assisted Extraction and Low-Pressure Chromatographic System with a Phenyl Monolithic Column for Amperometric Determination of Volatile α-Dicarbonyl Compounds in Coffee Brews

Abstract

In this chapter, a low pressure chromatographic system featuring a single 0.5 cm length phenyl monolithic column and an amperometric detector was developed to determine volatile α-dicarbonyl compounds in coffee brews. The analytical strategy relied on the extraction of the volatile α-dicarbonyl compounds and their derivatization with o-phenylenediamine, prior to the determination of the resulting quinoxaline derivative compounds in the designed chromatographic system. The studies of the mobile phase composition and electrochemical conditions are presented. The extraction of the volatile α-dicarbonyl compounds was performed resourcing to a recent approach based on the fan assisted extraction system. This system performs the full evaporation technique and allowed the quantitative recovery (> 85 %) of diacetyl and 2,3-pentanedione from the coffee brew based on the following experimental conditions: sample volume 30 μL, extraction period 15 min, acceptor solution (water, acetonitrile, 90:10, v/v) volume 400 μL, and sample temperature 50 °C. The developed method showed no matrix effect for different coffee brews and the quantification of the studied analytes can be performed through the external calibration method. Detection limits of 3.6×10^{-6} mol/L for diacetyl and 12.9×10^{-6} mol/L for 2,3-pentanedione were obtained. Sample analysis rate was of 5 h^{-1} and the acetonitrile consumption was of ca. 450 μL per chromatographic run.

B 2.1. Introduction

The α-dicarbonyl compounds (α-DC) found referred in roasted coffee include 3-deoxyglucosone [76], 2,3-pentanedione (PD) [208], and most commonly, GO, MGO, and DA [44,76,209]. α-DC are intermediate compounds of caramelization and Maillard reactions [210–212], and are considered relevant in terms of human toxicity and organoleptic properties. In this respect, some studies have focused their significance upon e.g. cellular damage [213], air quality [214,215], diabetes, and kidney failure [216]; additionally, DA and PD are considered key aroma impact compounds in roasted coffee [56] due to their well-known buttery and oily buttery odours [217], thereby justifying their determination in this matrix.

The methods described for the determination of α-DC in coffee samples typically include a derivatizing reaction with OPDA with formation of quinoxaline derivatives [44,76,211] combined with liquid–liquid extraction [44] or a clean-up step with SPE cartridges [76,209,211], before gas chromatography with a nitrogen-phosphorus detector (GC-NPD) [44] or HPLC-DAD [76,211] analysis. Other approaches for the determination of these compounds in food samples include the use of different derivatizing reagents e.g. 4-(2,3-dimethyl-6-quinoxalinyl)-1,2-benzenediamine [218] or 4-nitro-o-phenylenediamine [219], HPLC separation with phenyl based column [220], determination through fluorimetry [221] or electrochemical detectors [222].

In this work, a low-pressure chromatographic systems featuring for the first time a phenyl monolithic column was envisaged to perform the amperometric determination of volatile α-DC in coffee extracts. Low-pressure chromatographic systems with amperometric detection are a recent analytical strategy relying on a peristaltic pump, a short length monolithic column and the electrochemical detection system. It was originally exploited in the determination of vitamin B3 in brewed coffee samples [147] and additional analytical applications followed, namely, in the determination of CGAs and caffeine using partition chromatography [146], and in the parallel determination of oxidizable and reducible compounds by ion-pair chromatography using perfluoroheptanoic acid as ion-pair reagent [175]. This analytical approach showed potentiality to implement *ad-hoc* methods with high selectivity, high analysis throughput and low implementation cost. These features are a consequence of combining within conventional flow analysis systems, the efficiency of short-length monolithic columns with the potentialities of the electrochemical detectors. The use of phenyl columns is a novelty in this context. Phenyl columns are usually silica-based columns with phenyl

groups directly, or through an alkyl chain, bounded to the silica core thereby embodying the different types of phenyl columns currently available (e.g. phenyl–ethyl, phenyl-propyl or phenyl-hexyl). These columns provide a different chromatographic selectivity compared to the more usual alkyl C_n columns [223,224] as the former enable π-π interactions to be established between the phenyl group of the stationary phase and unsaturated poly(cyclic) aromatic molecules.

Furthermore, the analytical strategy herein delineated included an initial extraction step of the volatile compounds from the coffee brew to an acceptor solution containing a derivatizing reagent. The extraction step followed a recent strategy developed in our research group [225] based on the fan assisted extraction system as a mean to perform the full evaporation technique [226]. In brief, this pioneer extraction system is based on a closed flask (featuring an electric fan in the inner side of the lid), where the sample and the extraction solution are separately poured in. Under temperature-controlled conditions and by means of the strong convective effect promoted by the fan operation, faster evaporation of the volatile compounds of the sample occurs and a quantitative recovery of the analytes can be attained. The volatile compounds of the sample are extracted into the acceptor solution containing the derivatizing reagent OPDA (with formation of the respective quinoxaline derivatives) which are further determined in the low-pressure chromatographic system by amperometry.

B 2.2. Experimental

B 2.2.1. Reagents and Solutions

Deionized water (resistivity >18.2 MΩ cm, 0.2 μm filtered) was used in the preparation of aqueous solutions.

All reagents were used as purchased. GO (Sigma-Aldrich, ref.50649-25ML, 40 % in water), MGO (Sigma-Aldrich, ref.M0252-100ML, 40 % in water), DA (Sigma-Aldrich, ref. B85307-100ML, 97 %), and 2,3-pentanedione (Sigma-Aldrich, ref. 241962-100ML, 97 %) were used for the preparation of stock standard solutions, 1.00×10^{-2} mol/L, in ACN medium. Stock standard solutions of quinoxaline (Aldrich-Chemie, Q160-3, 99 %), 2-methylquinoxaline (Aldrich-Chemie, M8,020-2, 99 %), and 2,3-dimethylquinoxaline (Aldrich-Chemie, D18,497-7, 97 %), 1.00×10^{-2} mol/L, were prepared in water-ACN, 70:30 (v/v). All stock standard solutions were prepared each 3 weeks. Diluted solutions

of α-DC and quinoxaline derivative compounds were prepared by rigorous dilution in deionized water.

The derivatizing solution of 0.05 % (w/v) OPDA (Sigma-Aldrich, ref. 8145380250, ≥99 %) was prepared daily by dissolving 0.015 g of the reagent in 25 mL of 0.1 mol/L phosphate buffer solution (pH = 7.00) and kept in the dark. The phosphate buffer solution was prepared by dissolution of di-sodium hydrogen phosphate (ChemLab, ref.CL00.1436.100, >99.5 %) in deionized water followed by pH adjustment with a solution of 6.0 mol/L HCl.

For the cyclic voltammetry studies, the following supporting electrolytes were used: a solution of 0.01 mol/L HCl (pH = 2.00), prepared by dilution of 37 % w/w hydrochloric acid (Thermo Fisher Scientific, ref. H/1200/PB17); a buffer solution of pH = 4.50, prepared by dilution of acetic acid (VWR, ref. 20102.292, ≥99 %) to the concentration of 0.01 mol/L, followed by pH adjustment with a solution of 4.0 mol/L NaOH; a di-sodium hydrogen phosphate buffer solution pH = 7.00, prepared as abovementioned.

In the fan assisted extraction studies, the acceptor solution was prepared by mixing the derivatizing solution of OPDA and ACN, 90:10 (v/v).

For the chromatographic analyses in the low-pressure chromatographic flow system, the mobile phases were prepared by proper mixtures of ACN (Fischer Chemical, ref. A/0627/17) and deionized water or HCl solutions, the latter prepared by proper dilution of the abovementioned reagent.

B 2.2.2. Cyclic Voltammetry Studies

Cyclic voltammetry studies were performed for quinoxaline derivative compounds using a three electrodes system: the auxiliary electrode – a CGE rod (Metrohm, ref. 6.1247.000); the reference electrode– an Ag/AgCl double junction electrode (Thermo, ref. 90-02, internal solution: 3 mol/L KCl; external solution: 10 % (w/w) KNO_3 and 0.01 mol/L HCl) and the working electrode– a boron doped diamond electrode (Windsor Scientific, 3 mm ø, ref. 3MMDIAM.BDD.PEEK). The electrochemical measurements were performed using a potentiostat (μAutolab Type II) controlled through software GPES 4.9 (Eco Chemie, B.V., Utrecht, The Netherlands). The cyclic voltammograms were recorded between −1.5 V and +1.5 V, at a scan rate of 0.1 V/s, for model solutions of quinoxaline derivatives using different supporting electrolytes as referred in the previous section.

B 2.2.3 Low-Pressure Flow System Configuration and Operation

The low-pressure chromatographic flow system assembled consisted of a single channel manifold. It comprised a peristaltic pump (Gilson, Miniplus 3) featuring a tygon tubing of 1.02 mm i.d. (Gilson, Inc., F117938) to propel the mobile phase, an injection valve (Rheodyne, ref. 5020) featuring a 40 µL loop volume; a 0.5 cm length phenyl propyl monolithic column (Merck, Chromolith, ref. 1.52059.0001) inside a column holder (Merck, Chromolith, ref. 1.52255.0001), and a laboratory made poly(methyl methacrylate) confluence (Figure 14).

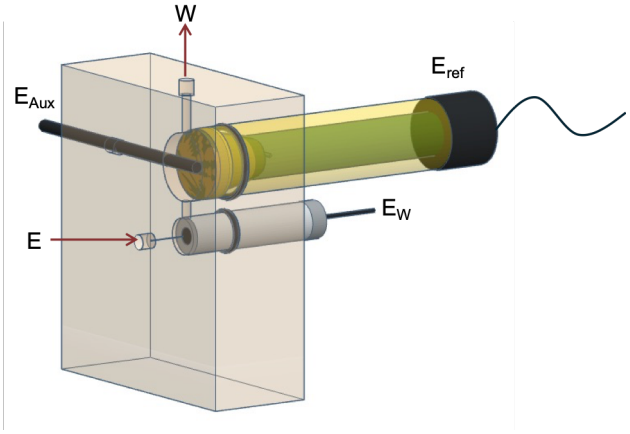


Figure 14 – Schematic representation of the lab-made confluence designs, with two inlet channels. E - Eluent; ISA - Ionic strength adjustment solution; W - Waste; E_{Ref} - Reference electrode; E_w - Working electrode; E_{Aux} - Auxiliary electrode.

The latter accommodated all three electrodes previously referred (Section B 2.2.2.) −the working electrode in a wall-jet configuration and featured an inlet and outlet channel. Through the inlet channel, the eluate was headed to the surface of the working electrode. The outlet channel was connected to the waste. For the remaining connections of the flow system, PTFE tubing 0.8 mm i.d. was used. A 3 m length tubing coil was connected at the flow system's exit to mimic a back pressure device and minimize air bubbles formation. The nylon syringe filter, 25 mm Ø, 1.0 µm (Whatman, 6750-2510), placed in the channel of the mobile phase served as in-line filter to extend the monolithic column lifetime, and was replaced each working week. As in previous works based on low-pressure chromatography systems with amperometric detection, the assembling of the manifold considered the least feasible distance between the electrodes (to reduce electrical noise), and between the monolithic column exit and the working electrode's surface (to reduce band broadening). A schematic representation of the system used is

presented in Figure 15 and the apparatus of the system used, as well as the monolithic column used is presented in Figure 16.

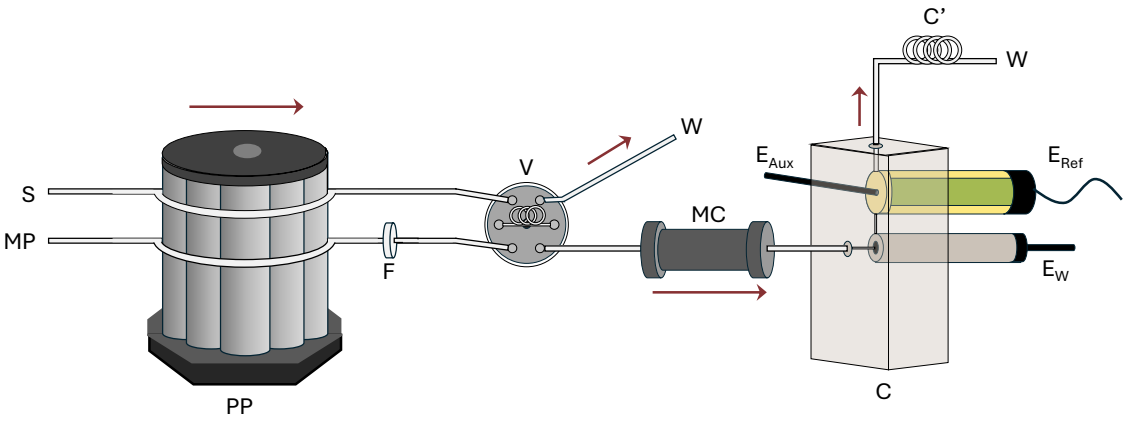


Figure 15 – Schematic representation of the low-pressure chromatographic system used. S – Sample; MP – Mobile phase; PP – Peristaltic pump; F – Filter; V – Injection valve; MC – Monolithic column; C – Lab-made confluence; E_W – Working electrode; E_{Aux} – Auxiliary electrode; E_{Ref} – Reference electrode; C' – coil.

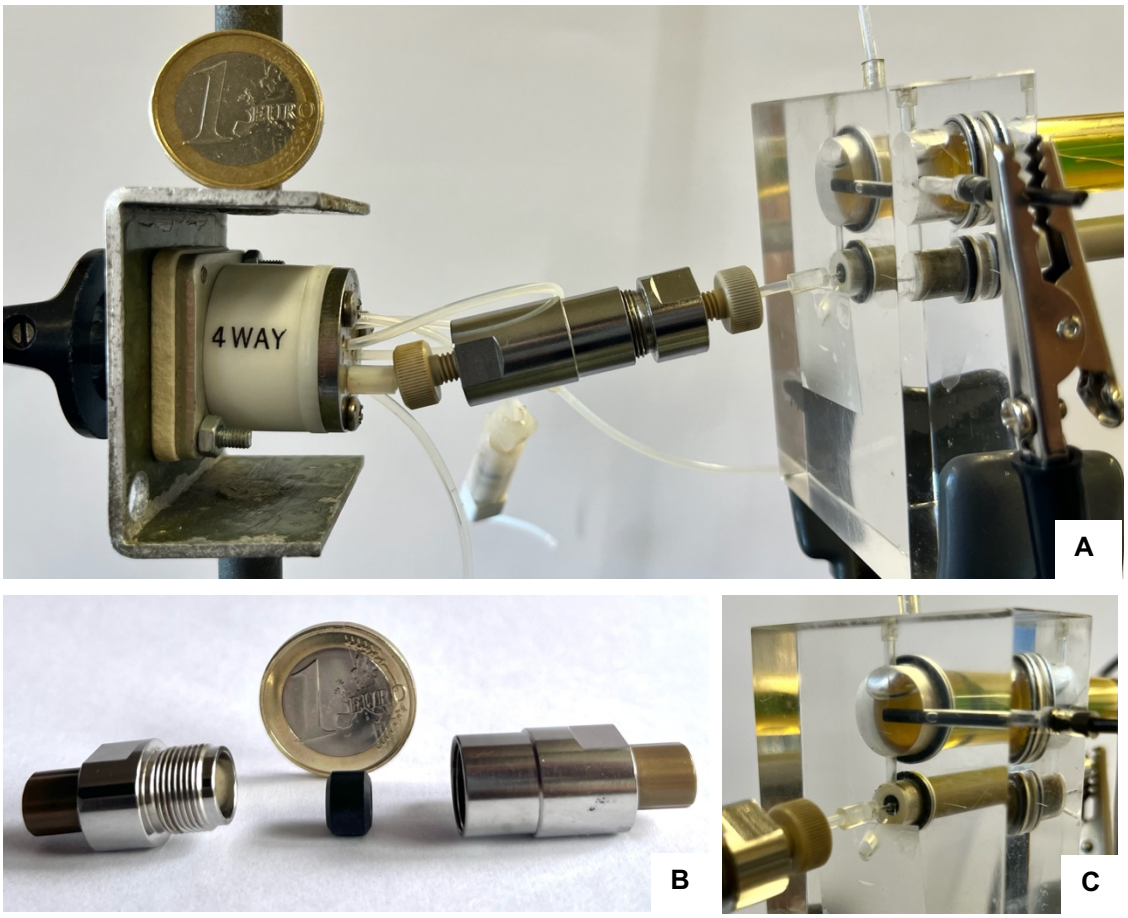


Figure 16 – Experimental apparatus of the low-pressure chromatographic flow system with amperometric detection. A – Low-pressure chromatographic flow system with amperometric detection; B – phenyl propyl monolithic column, with 0.5-cm length, and respective column holder; C – Lab-made confluence with the three electrodes system and one inlet.

The handling of the low-pressure chromatographic flow system followed usual procedures for flow analysis systems or liquid chromatographic systems. Based on previous works [146,148,175], the flow rate for the mobile phase was set to 0.70 mL/min. At the beginning of each working day, a stabilization period of 30 min was necessary for proper conditioning of the column and stabilization of the baseline current intensity. At the end of each working day, the monolithic column was conditioned with ACN to preserve its normal performance. Under the final experimental conditions selected in this work (the sample pre-treatment used provided a substantial matrix elimination), the monolithic column could be used for several months on a daily basis without significant loss of its performance.

B 2.2.4 Electrochemical Conditions and Mobile Phase Studies in the Low-Pressure Chromatographic System

The initial experimental studies aimed to obtain a stable cathodic electrochemical response toward successive injections of a 5.00 $\times 10^{-5}$ mol/L quinoxaline solution, in the low-pressure chromatographic flow system. Furthermore, the influence of OPDA in the electrochemical signal was assessed following the previous experimental procedure. To this end, a model solution containing GO, MGO, DA, and PD, at concentration of 5.00 $\times 10^{-5}$ mol/L, and 0.05 % (w/w) OPDA was prepared. This solution was left to stand during 40 min based on the well-known reaction period to obtain the quinoxaline derivative compounds [227] and thereafter injected in the low-pressure chromatographic system. In these preliminary studies, a mobile phase composition of 0.01mol/L HCl and ACN, 95:5 (v/v), was used. The acidification of the mobile phase resulted from previous studies with cyclic voltammetry (Section B 2.2.2.) being also the minimum pH value which the column withstands following the manufacturer indications; the selected low ACN content was based on previous expertise using short length columns in low-pressure chromatographic systems. Different potential values were then tested, using single pulse and MPA technique, until a stable and repeatable electrochemical response was achieved. In the next set of tests, one aimed to evaluate different compositions of the mobile phase to attain the chromatographic separation of the quinoxaline derivative compounds with the 0.5 cm length phenyl monolithic column. The mobile phase composition was studied considering different compositions of aqueous and organic phases. The mobile phase composition was selected according to the chromatographic resolution values (Rs) calculated.

B 2.2.5. Fan Assisted Extraction Apparatus and Operation

Based on a previous work [225], a fan assisted extraction system was assembled to extract the α-DC from coffee brews. Briefly, this system comprised a closable flask (Schott, 218012458, 100 mL) with an electric fan (Coolerguys, 25 ×25 ×10 mm, IP67, 12 V) in the inner part of the lid, and a cylinder-shaped PTFE reservoir (43 mm h × 20 mm Ø) featuring a cavity in the top surface for accommodation of the acceptor solution. A schematic representation of the system used is presented in Figure 17.

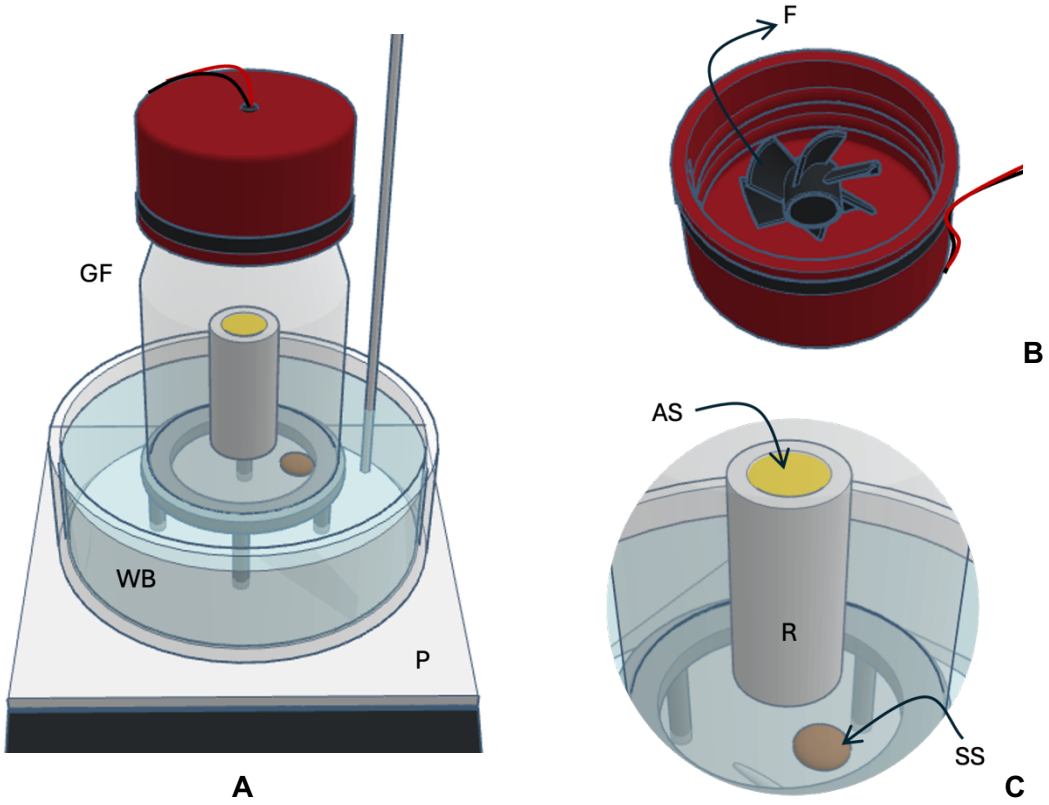


Figure 17 – Schematic representation of the fan assisted extraction system. GF – Glass flask (100 mL); WB – Water bath; P – Heating plate; F – Electric fan (4.5 V); R – PTFE reservoir; AS – Acceptor solution; SS – Sample solution.

The experimental procedure for the extraction was similar to the one originally referred: a rigorous volume of coffee brew was transferred to the empty flask; thereafter, the PTFE reservoir was placed inside the flask and a defined volume of the acceptor solution (400 µL) rigorously transferred into the reservoir's cavity. The flask was closed and placed in a water bath heated by a heating plate for temperature control. The fan was then switched on for a defined period, during which the extraction occurs. At the end of the extraction time, the fan was switched off and the acceptor solution collected to be analysed in the low-pressure chromatographic system. Between the extraction

procedures, the flask and the PTFE reservoir were washed with a solution of water and ACN, 50:50 (v/v), and dried before further use. The lid with the electric fan was placed in another 100 mL flask with approximately 10 mL of water and ACN, 50:50 (v/v), and turned on for 10 min to prevent contaminations between sample analyses.

The acceptor solution composition was evaluated considering its content in ACN and the respective impact in the chromatographic elution. Mixtures of water and ACN (90:10, 75:25, and 50:50 (v/v)) were prepared and tested for three injection volumes (10, 20, and 40 µL). Thereafter, studies using the fan assisted extraction system were conducted aiming to evaluate the recovery yield of the α-DC compounds (GO, MGO, DA, and PD) from an aqueous model solution, 1.00×10^{-4} mol/L. The following parameters were studied: time of extraction (5, 10, 15, and 20 min), and volume of sample (10, 20, and 30 µL). Recovery yield values were calculated considering as 100 % signal, the direct analysis of the acceptor solution with addition of the sample volume (bypassing the fan assisted extraction procedure and performing the volume correction inherent to each extraction period condition).

B 2.2.6. Coffee Samples and Brewing Procedure

A blended coffee (a mixture of arabica and robusta species), roasted and milled, was purchased from the local market to embody a mainstream consumed coffee. Coffee brewing was performed by the three most conventional procedures, decoction, espresso and infusion [41]. Deionized water and the same mass of coffee was used, 5.80 g, for all brews. In the decoction procedure, 15 mL of boiling water were poured over the coffee and left under gentle stirring for 2 min. Espresso coffee was obtained using a Nespresso® coffee machine, after pre-setting the brew volume to 15 mL. Infusion coffee was obtained using a conventional pour-over coffee maker, through which 15 mL of boiling water were poured over the filter paper containing the coffee and left to drip during aprox. 2 min. All brews were prepared/collected directly into closeable flasks. At the end of each brewing procedure, final volumes were immediately adjusted to 15.0 mL by weighing and the flasks sealed to avoid volatile compounds loss. Chromatographic analyses were performed after samples reached room temperature.

B 2.2.7. Calibration Curves

The following experimental conditions were used in both external calibration method and standard addition method: the extraction procedure using the fan assisted system was performed with a sample volume of 30 µL, an acceptor solution volume of

400 µL (a mixture of 0.05 % (w/w) OPDA solution, ACN, 90:10 (v/v)), during 15 min, and the water bath temperature was set at 50 °C. The chromatographic analyses were performed using a mobile phase composition of 0.0056 mol/L HCl and ACN, 95:5 (v/v). Loop volume of the low-pressure chromatographic system was of 40 µL. Electrochemical detection was performed through MPA (E_{red} = − 1.0 V (0.03 s), E_{cond} =+2.8 V (0.05 s) and E_{cond2} = +1.4 V (0.20 s)).

The calibration curve by the external calibration method was attempted using standard solutions with equimolar concentrations of DA and PD within the range 2.00×10^{-5} to 8.00×10^{-4} mol/L.

The standard addition method was conducted as follows: four spiked brew coffee solutions were prepared with the following added concentrations of the referred compounds 5.00×10^{-5}, 1.00×10^{-4}, 1.50×10^{-4}, and 2.00×10^{-4} mol/L. Each spiked brew coffee sample was submitted to the fan assisted extraction system and the respective extract analysed using the designed low-pressure chromatographic flow system.

A statistical comparison [228] between the slopes from the calibration curve with the slopes of the standard addition method, for the three brewed coffee samples, was conducted to evaluate sample's matrix effect upon the studied analytes.

B 2.3. Results and Discussion

B 2.3.1. Cyclic Voltammetry of Quinoxaline Derivatives

The preliminary tests to study the electrochemical behaviour of quinoxaline derivative compounds in different pH conditions, at the boron doped diamond electrode, showed faradaic electrochemical signals of increasing intensity under more acidic conditions along the anodic and cathodic sweeps (Figure 18).

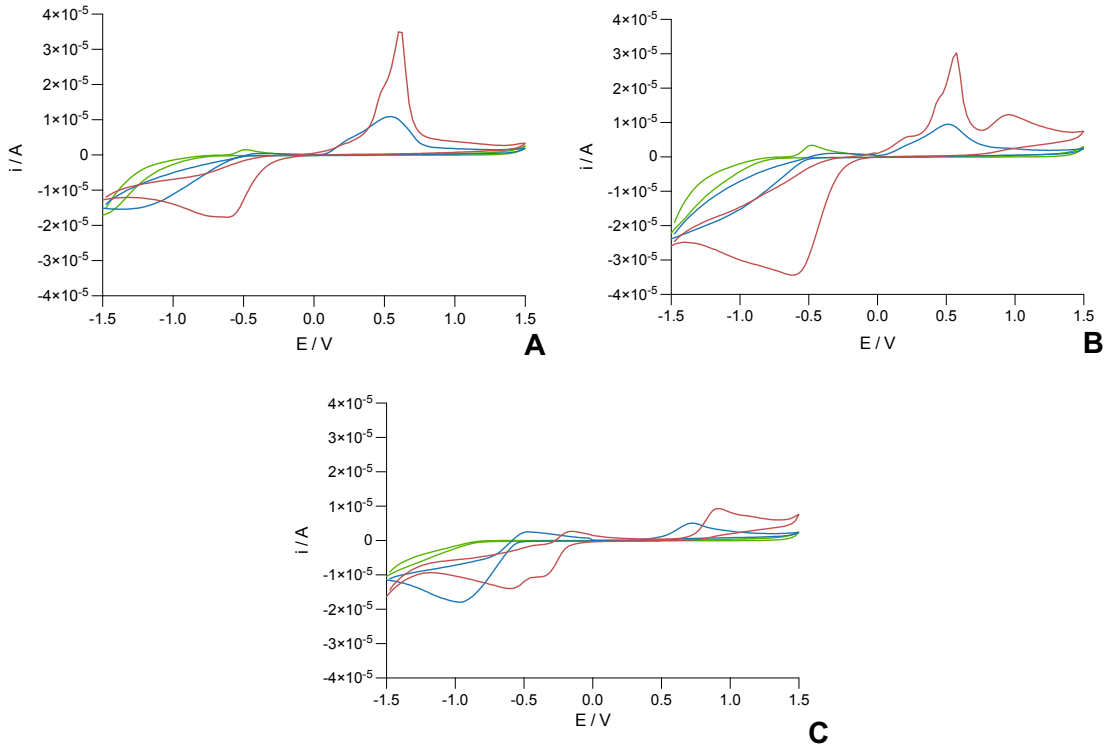


Figure 18 – Cyclic voltammograms at different pH conditions for (A) quinoxaline, (B) 2-methylquinoxaline, and (C) 2,3-dimethylquinoxaline, 1.00 ×10^{-3} mol/L. Electrochemical conditions: scan rate 100 mV/s, E_0 =0.0 V; First vertex = −1.5 V; Supporting electrolyte as referred in Section B 2.2.1.: (—) pH 2.00; (—) pH 4.50 and (—) pH 7.00.

To the best of our knowledge, two works about the electrochemical behaviour of quinoxaline at a solid electrode (GCE) were found [229,230]. Although there is no consensus in the cyclic voltammograms profile from the referred works, the impact of the supporting electrolyte upon the cyclic voltammograms profile [230] and a discussion of the redox mechanisms involved are shown [229,230]. In this work, the anodic signal intensity observed at ca. 0.6 V (pH = 2.00) decreased for less acidic conditions and can only be seen after previous cathodic sweep likewise referred in the literature [229]. The anodic signal observed at ca.−0.5 V was also reported at the GCE, although in our study it decreased its intensity under less acidic conditions.

The results obtained also showed the impact of the methyl groups in the cyclic voltammetry profile, nevertheless a deeper understanding of the electrochemical processes for 2-methylquinoxaline and 2,3-dimethylquinoxaline is outside the scope of this work. For the case being, all studied analytes presented a cathodic signal at E < −0.5 V in acidic conditions and, therefore, further experiments taken these results into consideration.

B 2.3.2 Results for the Electrochemical Conditions and Mobile Phase Composition Studies in the Low-Pressure Chromatographic Flow System

Preliminary results after successive injections of a model solution of quinoxaline showed no stable reduction signal at the boron doped diamond electrode (E_{red} = −1.0 V). Likewise observed in previous works [148,175], an additional anodic potential pulse for conditioning of the electrode was required. Using MPA, a stable response was achieved using the following electrochemical conditions E_{red} = −1.0 V (0.03 s) E_{cond} = +1.0 V (0.10 s), a similar behaviour to the one described in the work published by Sousa et al. [148]. Further experiments with solutions containing α-DC in the presence of OPDA showed, however, a profound change in the baseline stability (Figure 19A). Likely cause is the well-known oxidation of OPDA to the corresponding oligomers and polymers at solid electrode's surface [231] which found analytical applications in the synthesis of molecularly imprinted polymers [232,233]. To overcome the baseline instability, an option was made by progressively increase the E_{cond} value (0.1 V increments were evaluated), until complete mitigation of the OPDA signal interference. A value of E_{cond} = +2.8 V (0.10 s) accomplished this purpose. Concomitantly, it was also observed an increase of the baseline noise due to the evolution of dioxygen at the electrode's surface and also due to the high potential amplitude and fast potential change between the two potential pulses imposed. Therefore, the period of E_{cond} = +2.8 V was reduced to 0.05 s and an additional potential pulse (E_{cond2}) was lastly evaluated. For E_{cond2}, a period of 0.20 s was selected, still enabling a reasonable sampling rate (ca. 3.6 measurements *per* second). Decrements of 0.1 V potential values starting from +2.7 V, revealed a stable baseline for E_{cond2} = +1.4 V and an amplitude signal of 0.3 μA, which is within the order of magnitude observed in previous works operating under a two-pulse condition. Final selected MPA conditions were then E_{red} = −1.0 V (0.03 s), E_{cond} = +2.8 V (0.05 s) and E_{cond2} = +1.4 V (0.20 s).

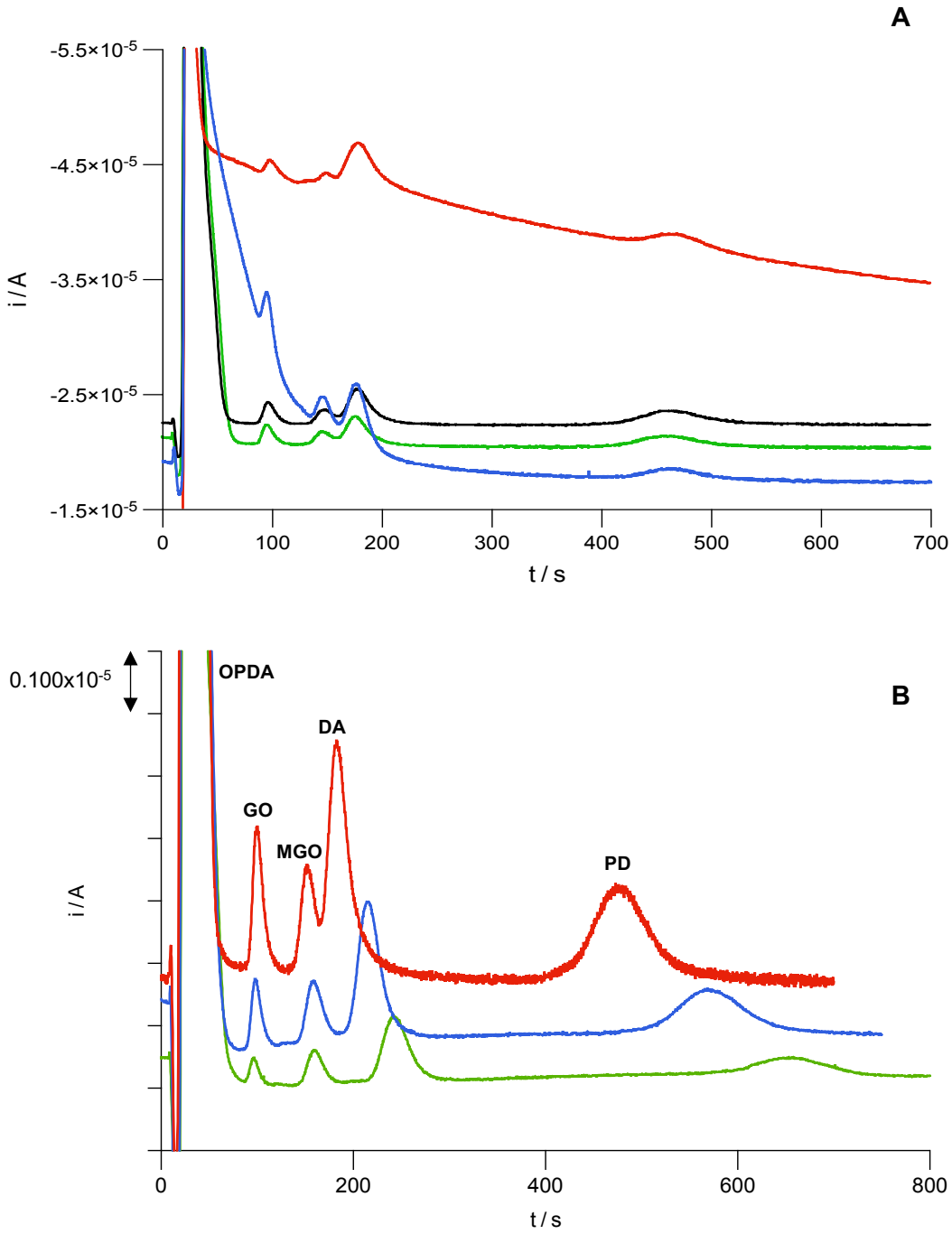


Figure 19 – (A) Chromatograms obtained using E_{red} = −1.0 V (0.03 s) and different conditioning electrochemical conditions for a mixture of α-DC, 5.00 × 10^{-5} mol/L, and OPDA, 0.05 % (w/v): (—) E_{cond} = +1.0 V (0.10 s); (—) E_{cond} = +2.0 V (0.10 s); (—) E_{cond} = +2.8 V (0.03 s) E_{cond2} = +1.4 V (0.10 s); (—) E_{cond} = +2.8 V (0.05 s) E_{cond2} = +1.4 V (0.20 s). (B) Chromatograms obtained using E_{red} = −1.0 V (0.03 s) E_{cond} = +2.8 V (0.05 s) E_{cond2} = +1.4 V (0.20 s) and the following mobile phase compositions: HCl solution (—) pH 2.00; (—) pH 2.25; (—) pH 2.50 and ACN, 95:5 (v/v).

For the following mobile phase solutions containing 0.01 mol/L HCl and ACN, 95:5, 96:4, 97:3, and 98:2 (v/v), one observed a general increase of the retention times of α-DC for mobile phases with less content in ACN as expected. Nevertheless, a quantitative separation of the chromatographic peaks was not attained. Therefore, one exploited the

impact of changing the pH of the mobile phase taking into account the different acidity constants of the quinoxaline derivatives (pKa = 0.56, 1.32, and 1.69, for quinoxaline, 2-methylquinoxaline, and 2,3-dimethylquinoxaline, respectively; pKa data for 2-ethyl-3-methylquinoxaline was not found). As foreseen, a slightly less acidic media enabled the quinoxaline derivatives with higher pKa to be more retained (Figure 19B). The values of the chromatographic parameters are presented in Table 12.

Table 12 – Chromatographic parameters for the peaks of quinoxaline derivative compounds in the low-pressure chromatographic system using different mobile phases.

	HCl 0.01 mol/L (pH 2.00), ACN, 95:5 (v/v)				HCl 0.0056 mol/L (pH 2.25), ACN, 95:5 (v/v)				HCl 0.0032 mol/L (pH 2.50), ACN, 95:5 (v/v)			
	GO	MGO	DA	PD	GO	MGO	DA	PD	GO	MGO	DA	PD
Rt, s	99	152	183	476	98	159	216	569	96	159	243	649
Rs*	1.94	0.82		8.35	1.87	1.50		8.65	1.44	1.92		11.48
Asymmetry	1.69	1.60	1.33	1.18	1.54	1.20	1.29	1.23	1.40	1.31	1.39	1.28
H, (10^{-5} m)	1.47	1.48	1.62	1.79	1.31	1.28	1.29	1.67	1.10	1.39	1.51	0.948

*Considering the closest peak.

A mobile phase composition of 0.0056 mol/L HCl, and ACN, 95:5 (v/v), was selected as a compromise situation which enabled full separation of the chromatographic peaks without significantly decrease their signal intensity (mobile phase with a lower pH would provide higher sensitivity). The values observed for asymmetry and plate number are within typical values considering similar manifolds [146,148].

B 2.3.3 Extraction of Volatile α-DC Using the Fan Assisted Extraction System

Based on the higher solubility of 2,3-pentanedione in organic solvents (which advised the preparation of the acceptor solution using a water:ACN mixture), and taking into consideration that injection of samples in ACN media may disturb the elution of the chromatographic run (due to the column's low dead volume and the low content of ACN in the mobile phase), a preliminary experiment was carried out to evaluate the impact of different volumes of acceptor solution prepared with different ACN water ratios in the elution process. Mixtures of water and ACN, 90:10, 75:25, and 50:50 (v/v), were then tested considering loop injection volumes of 10, 20, and 40 µL. For the solution with the higher ACN content, disturbance in retention time values was always observed. For the mixture water:ACN 75:25 (v/v), a shift in the elution process was observed for 20 µL and 40 µL of injection volume. Considering the maximum loop volume tested, the mixture

water:ACN, 90:10 (v/v) could be used. The starting experimental conditions to study the recovery yield using the fan assisted system were conducted using the latter conditions.

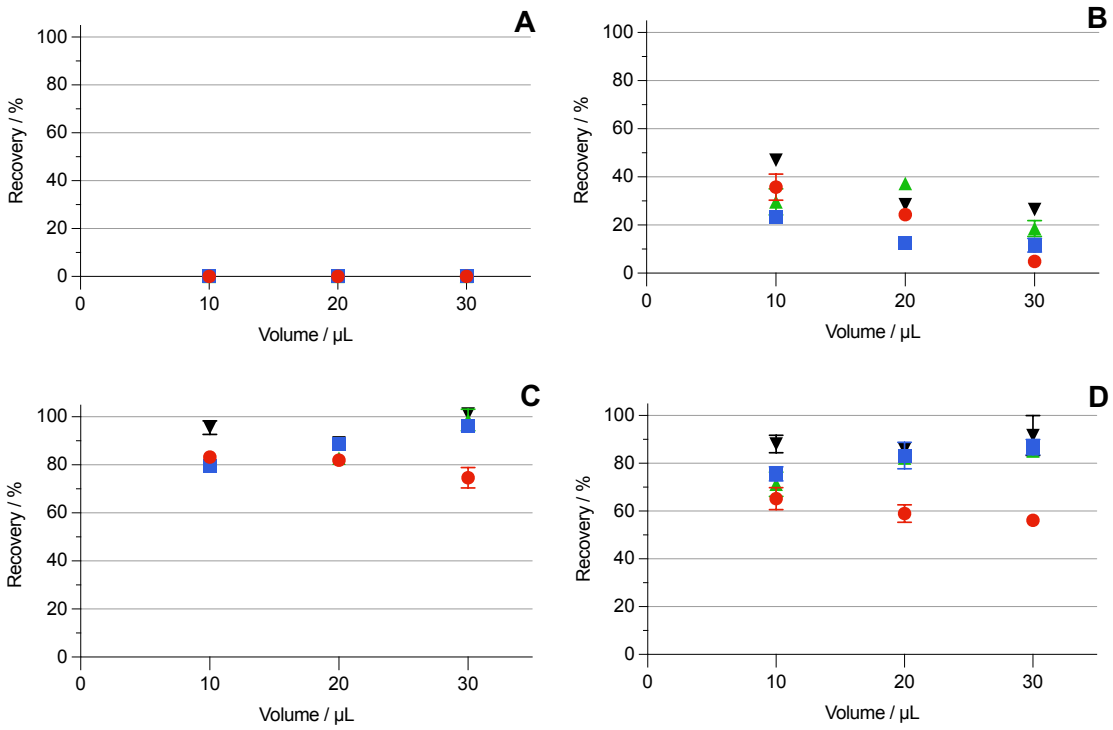


Figure 20 – Recovery values using the fan assisted extraction system for sample volumes containing α -DC, 1.00 ×10^{-4} mol/L. Quinoxaline derivative compounds of (A) – GO, (B) – MGO, (C) – DA, and (D) – PD; (●) – 5 min, (■) – 10 min, (▲) – 15 min, (▼) – 20 min; RSD < 6 % (n=2).

The results of recovery yield for DA and PD using the fan assisted extraction system showed the following dependence toward the experimental parameters studied: within the extraction periods tested (5, 10, 15, and 20 min) higher recovery yields were obtained for longer periods; within the sample volumes interval tested (10, 20, and 30 µL), higher peak area values could be obtained for larger sample volumes but no difference was observed in the recovery yield (Figure 20). The recovery yield profile was similar the one reported in the original work and the best results were achieved for extraction periods ≥10 min and sample volume of 30 µL, which enabled recoveries higher than 95 % for DA and 85 % for 2,3-pentanedione. Of notice, the absence and the very low recoveries for GO and MGO, respectively. An explanation for this fact may be the reactivity of these compounds as found reported [234,235]. In aqueous media, GO and MGO are prone to be hydrated and to form dimers hindering their evaporation. Furthermore, the analysis of the acceptor solution also revealed peaks of small intensity only at the retention times of MG and MGO. These peaks were identified as degradation products of the derivatizing reagent, since heating of the acceptor solution occurred

during the fan assisted extraction process. In this scenario, no further studies were performed regarding the extraction conditions and compounds GO and MGO were not considered for quantification purposes.

B 2.3.4 Evaluation of the Developed Method

The calibration curves with model solutions of DA and PD using the experimental conditions referred in previous sections (B 2.3.2 and B 2.3.3) showed a linear relationship between the concentrations tested for each studied compound versus the respective peak areas. The figures of merit obtained are presented in Table 13.

Table 13 – Typical figures of merit[#] for quinoxaline derivative compounds of DA and 2,3-pentanedione using the fan assisted extraction system and the low-pressure chromatographic flow system.

		DA	PD
Calibration curve equation[§]	Slope, (A L mol^{-1})	6.73 (±0.38) x 10^{-2}	5.85 (±0.33) x 10^{-2}
	Intercept, A	3.93 (±1.28) x 10^{-6}	2.09 (±1.12) x 10^{-6}
Calibration range, (mol/L)		(2.0 to 80.0) x 10^{-5}	
Coefficient of correlation		0.9999	0.9981
Limit of detection[#], (mol/L)		3.6 x 10^{-6}	1.29 x 10^{-5}
RSD intraday[£]		2.1	2.7
RSD inter day*		6.4	7.1
Analysis rate[$], h^{-1}		ca. 5	
ACN consumption[&], (µL/analysis)		450	

[#] - calculated according to [236].

§ Values with confidence limits.

£ n =3.

* n =2, for 3 consecutive days.

$ 15 min for the volatile compounds extraction step not considered.

& 40 µL in the acceptor solution +ca. 410 µL *per* chromatographic run.

The detection limit achieved for DA was suitable for its determination in coffee samples, based on typical values found reported [44,211]. No published works regarding the concentration of 2,3-pentanedione in brewed coffee samples were found.

The values of RSD intraday (<3 %), analysis rate (ca. 5 h^{-1}) and ACN consumption *per* analysis (448 µL) were also within the typical ones previously reported in low-pressure chromatographic systems [146,148,175].

Calibration curves using the standard addition method were also performed for brewed coffee samples prepared by infusion, decoction and expresso techniques. The statistical comparison of the slopes obtained by the standard addition method respective to each of the brews prepared versus the slope of a calibration curve obtained with standard solutions, revealed differences not significant (the values of test F and t-student calculated are presented in Table 14. These results show that performing the fan assisted extraction method mitigates the coffee matrix effect and enables the quantification of the studied analytes by the external calibration method, likewise verified previously [225].

Table 14 – Statistical tests for slopes comparison between standard addition method in different coffee brewing procedures versus external calibration curve*

α-DC	Espresso method		Infusion method		Decoction method	
	F-test	*t*-test	F-test	*t*-test	F-test	*t*-test
DA	5.15	0.19	5.23	2.18	7.71	1.68
PD	10.44	0.22	1.60	0.73	1.50	1.26

t_{critic} = 2.45 (95 %, υ = 6); F_{critic} = 15.44 (95 %, υ_1 and υ_2 = 3)

* - statistical tests performed as referred in [228].

Typical chromatograms obtained for coffee samples prepared according to the decoction brewing method with standard additions of DA and 2,3-pentanedione are presented in Figure 21. The same chromatogram profile was observed for espresso and infusion brewing procedures. Two additional chromatographic signals were observed (P2 and P3). These peaks are degradation products of OPDA as previously referred, and P3 can also have a contribution of MGO compound based on its low recovery under the fan assisted conditions. Further study of these peaks was outside the scope of this work but it is worth noticing the chromatographic performance of a single 0.5 cm length phenyl column under the selected experimental conditions.

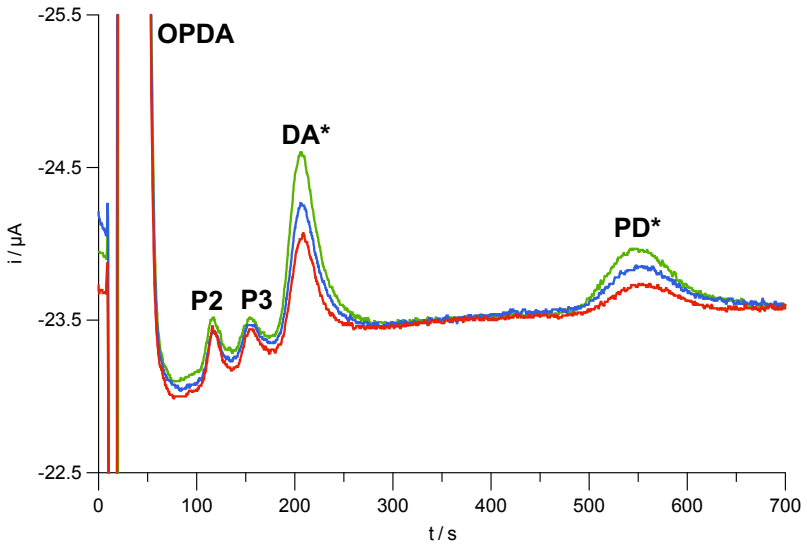


Figure 21 – Typical chromatogram of a coffee extract prepared by decoction method, and analysed using the developed methodology: without standard addition (—), spiked with 1.00 ×10^{-4} mol/L (—) and 2.00 ×10^{-4} mol/L (—) of DA and PD. P2 and P3 are non-identified peaks. * − Quinoxaline derivative peaks of DA and PD.

The concentrations found for DA were 7.0 (±1.5) × 10^{-5}, 5.1 (±0.1) × 10^{-5}, and 4.6 (±0.3) × 10^{-5} mol/L, for espresso, infusion and decoction brewed samples, respectively. The concentrations found for PD were 4.3 (±0.7) × 10^{-5}, 4.5 (±0.2) × 10^{-5}, and 3.9 (±0.2) × 10^{-5} mol/L, for espresso, infusion and decoction brewed samples, respectively.

The selectivity of this *ad-hoc* analytical system is supported by the inherent experimental conditions used, i.e., the sample pre-treatment procedure enables only the extraction of volatile compounds to the acceptor solution (a low temperature was used in this procedure, 50 °C), with remaining coffee matrix compounds standing at the bottom's flask; the use of a phenyl based monolithic column in the low-pressure chromatographic flow system is more suitable for separation of aromatic compounds and; the electrochemical conditions selected only focus electroactive compounds at the boron doped diamond electrode and able to be reducible at potential values lower than the selected E_{red} potential.

Furthermore, the selectivity of the developed analytical method is also supported by the derivatizing reagent selected, OPDA, which is highly selective toward α-dicarbonyl compounds. In fact, an experimental trial with brewed coffee samples, where the fan assisted extraction procedure was conducted using an acceptor solution without OPDA, retrieved no chromatographic peaks at the retention times of quinoxaline derivatives of DA and PD. This simple experimental test is illustrative of the absence of additional

coexisting substances in brewed coffee samples under the experimental conditions used.

In addition to the abovementioned, current literature already screens the main α-dicarbonyl compounds present in roasted coffee. These compounds, previously referred in the introduction section, are 3-deoxyglucosone, PD, GO, MGO, and DA. 3-deoxyglucosone is not volatile, therefore not extracted under the experimental conditions used, and the remain referred compounds were properly evaluated and discussed along the work developed.

Lastly, a comparison of some features of the developed methodology versus recent analytical methodologies found reported for determination of α-DC in coffee matrix, is resumed in Table 15.

The competitiveness of this *ad-hoc* analytical approach is present in its inherent low cost of implementation and operation, and the ability to determine PD. Reported methodologies, particularly the ones based on HPLC systems, typically require the use of SPE cartridges and can determine GO and MG compounds. Furthermore, the presented methodology presents higher analysis rate and lower consumption of organic eluents.

Table 15 – Features of recent analytical methodologies for the determination of α-dicarbonyl compounds in coffee matrices.

α-dicarbonyl compounds[&]	Matrix	Sample pre-treatment		Analytical technique				Year of publication, reference
		Procedure	Time	Instrument	Analysis time	Detection limit of DA	Waste generation	
GO, MGO, DA	Brewed green and roasted coffee	SPE and derivatization	3 h derivatization time	HPLC-DAD	72 min	0.002 µg/mL	~15 mL ACN*	2007, [211]
GO, MGO, DA	Brewed roasted coffee	SPE and derivatization	3 h derivatization time	HPLC-DAD	72 min	----	~15 mL MeOH*	2007, [209]
DG, GO, MGO, DA	Brewed roasted coffee	SPE and derivatization	1 h derivatization time	HPLC-DAD	47 min	0.001 µg/mL	~6 mL MeOH	2014, [76]
GO, MGO, DA	Brewed roasted coffee	Derivatization and LLE	2 h derivatization time	GC-NPD	38 min	0.19 µg/mL	----	2021, [44]
DG, GO, MGO, DA	Brewed roasted coffee	Derivatization	20 h derivatization time	UPLC-MS	9 min	----	~1 mL ACN	2021, [210]
GO, MGO, DA	Brewed roasted coffee	Derivatization and LLE	2 h derivatization time	GC-NPD	38 min	0.16 µg/mL	-----	2021, [78]
GO, MGO, DA	Brewed roasted coffee	Derivatization	50 min derivatization and extraction time	HS-SPME-GC-MS[$]	25 min	0.04 µg/mL	----	2023, [237]
GO, MGO, DA	Brewed roasted coffee	Derivatization	50 min derivatization and extraction time	HS-SPME-GC-MS[$]	40 min	0.19 µg/mL	----	2024, [238]
DA, PD	Brewed roasted coffee	Fan assisted extraction and derivatization	40 min extraction and derivatization time	LPC-ED[#]	12 min	0.31 µg/mL	~0.4 mL ACN	This work

$ HS-SPME-GC-MS, head-space solid phase microextraction with gas-chromatography and mass detection

LPC-ED, low-pressure chromatography with electrochemical detection

& DG, 3-deoxyglucosone; PD, 2,3-pentanedione.

B 2.4 Conclusions

An analytical method for the determination of volatile α-DC in coffee brews was developed. The analytical method comprised an initial extraction and derivatization step of the volatile compounds using a fan assisted extraction system, followed by the determination of the derivatized compounds using a low-pressure chromatographic system featuring, for the first time, a single 0.5 cm short length phenyl based monolithic column and an amperometric detector.

The recovery yield values using the fan assisted extraction system were similar to the ones referred in the original work presenting this strategy, thereby illustrating the applicability of this approach for quantitative extraction of volatile α-DC.

The experimental conditions selected in terms of mobile phase composition and electrochemical potential values enabled full separation of the targeted analytes and mitigation of the electrochemical signal disturbance derived from the derivatizing reagent used. The methodology developed showed the good performance of the low-pressure chromatographic system, in terms of selectivity, repeatability, sample analysis rate, and organic solvent consumption in spite of its very simple design and implementation cost. These are appealing features compared to usual figures of merit of conventional HPLC systems and encourage not only the development of further analytical methods based on this approach but also the use of this method to determine these analytes in other matrices.

PART C

A Green Approach to Ultrasound Assisted Dispersive Liquid-Liquid Microextraction

Synopsis

Part C of this PhD thesis focussed on the application of ultrasound assisted dispersive liquid-liquid microextraction (US-DLLME) with GC-MS analysis for the determination of volatile organic compounds in different food matrices. These two chapters present developments in US-DLLME-based sample preparation at improving the greenness of the technique without compromising analytical performance.

Chapter C 1 explored the determination of targeted carbonyl compounds and untargeted volatile organic compounds (VOCs) in adult nutritional formulas stored under various temperature conditions. The analytical methodology for sample preparation integrated US-DLLME for extraction and derivatization of targeted carbonyl compounds and headspace solid-phase microextraction for untargeted VOC monitoring. The developed methodology demonstrates high precision, linearity, low detection limits, and low solvent consumption. Furthermore, this work enabled the assessment of thermally induced carbonyl compounds and showed how storage conditions influence the carbonyl composition in adult nutritional formulas, highlighting the importance of analytical monitoring for quality control in these products.

In **Chapter C 2**, the aim was to improve the sample preparation strategy implemented in the previous work (US-DLLME), for the determination of carbonyl compounds in coffee samples. The goals focused on evaluating greener solvents and reduced extraction volumes to develop a more sustainable sample preparation approach. Following multivariate experimental designs, low-density extraction solvents and EtOH dispersive solutions were evaluated to optimize the extraction and derivatization steps. The greenness of the developed methodology was assessed through the AGREEprep tool. The results demonstrated that environmentally conscious solvent selection can be compatible with efficient extraction and reliable quantification, supporting the alignment of analytical methodologies with green chemistry principles.

Chapter C 1 was adapted from the following paper:

*Custodio-Mendonza, J. A.; **Silva, A. R**.; Kurek, M. A.; Almeida, P. J.; Santos, J. R.; Rodrigues, J. A.; Carro, A. M. Assessment of Lipid Peroxidation Products in Adult Formulas: GC-MS Determination of Carbonyl and Volatile Compounds Under Different Storage Conditions*

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Chapter C 2 was adapted from the following paper:

***Silva, A. R**.; Custodio-Mendonza, J. A.; Santos, J. R.; Almeida, P. J.; Rodrigues, J. A.; Carro, A. M. Green solvents in dispersive liquid-liquid microextraction for the determination of carbonyl compounds in coffee extracts*

Manuscript published in Journal of Chromatography A

DOI: 10.1016/j.chroma.2025.465743

Chapter C 1

Assessment of Lipid Peroxidation Products in Adult Formulas: GC-MS Determination of Carbonyl and Volatile Compounds Under Different Storage Conditions

Abstract

The occurrence of carbonyl compounds and volatile organic compounds (VOCs) in adult formulas is a critical issue in product safety and quality. This chapter reports the determination of targeted and untargeted carbonyl compounds and VOCs in adult formulas stored at different temperatures (room temperature, 4 °C, and 60 °C) over one month. Gas chromatography-mass spectrometry was utilized for the sample analysis. Ultrasound-assisted dispersive liquid–liquid microextraction at 60 °C for 20 min facilitated the extraction of six carbonyl compounds, while headspace solid-phase microextraction (HS-SPME) was employed for the determination of untargeted VOCs using a DVB/CAR/PDMS fibber, involving 15 min of equilibration and 45 min of extraction at 40 °C with magnetic stirring. Analytical features of the methods were assessed according to Food and Drug Administration guidelines, and good limits of detection and quantitation, linearity, accuracy, and precision were achieved. Notably, the highest levels of carbonyl compounds were found in high-protein formulas, with quantifiable levels of malondialdehyde, acrolein, and formaldehyde detected and quantified in 80 % of samples. Additionally, significant levels of VOCs such as hexanal and 2-heptanone were found in samples stored at elevated temperatures. These findings suggest the importance of protein content and storage conditions in the levels of carbonyl compounds and VOCs found in adult formulas, with implications for consumer safety and quality control.

C 1.1 Introduction

Adult formulas are specially designed nutritional products for enteral feeding, providing essential nutrients to individuals who may have difficulty consuming regular food [239,240].These formulas play a vital role in the nutrition and health of various adult populations, particularly those with chronic illnesses, disabilities, or other health problems requiring specialized dietary support [240,241]. For instance, patients recovering from surgery, individuals with malabsorption issues, and the elderly often rely on these products to meet their nutritional needs and improve their overall health outcomes [239–241]. Stringent regulatory standards govern the manufacturing of adult formulas to ensure safety and efficacy [242]. Formulations can vary widely, including standard, high-protein, and specialized options tailored for specific health conditions [241,242]. Ultra-high temperature treatment, commonly used in adult formula processing, rapidly heats products to 135–150 °C for a few seconds to destroy harmful microorganisms and extend shelf life without refrigeration [243]. However, this process can also lead to the formation of heat-induced contaminants, such as lipid peroxidation products and advanced glycation end-products, which may pose health risks to consumers [244].

Among the most concerning contaminants are malondialdehyde (MDA), formaldehyde (FCHO), acetaldehyde (ACE), acrolein (ACRL), MGO, DA (the structural formula is provided in Figure 22), and other various volatile organic compounds (VOCs) [245]. These compounds are formed during lipid peroxidation as polyunsaturated fatty acids interact with reactive oxygen species [244,245]. This process follows a radical-driven pathway, forming lipid hydroperoxides as primary oxidation products [245]. These hydroperoxides decompose into more stable secondary products, including carbonyl compounds such as MDA, FCHO, ACE, ACRL, MGO, and DA [245]. These compounds indicate oxidative degradation in lipid-containing foods like adult formula [244,245]. Lipid peroxidation can continue when the formulas are stored along with other degradation processes, potentially affecting their nutritional quality and safety [244,245]. Monitoring the presence and concentrations of these contaminants is crucial due to their toxicological profiles and classifications by health authorities, including the International Agency for Research on Cancer, which identifies several of these compounds as potential carcinogens [245]. For instance, FCHO is classified as a carcinogen with sufficient evidence of its carcinogenicity to humans (Group 1) with a tolerable daily intake set at 150 µg/kg body weight (bw)/day. ACRL is classified as probably carcinogenic to humans (Group 2A), with a lower tolerable daily intake of 7.5 µg/kg bw/day. ACE is categorized as possibly carcinogenic to humans (Group 2B), suggesting it may be

carcinogenic, with an acceptable daily intake of 185 µg/day. MDA falls into the group of substances with insufficient evidence for their carcinogenicity (Group 3). However, it possesses a Threshold of Toxicological Concern set by the International Programme on Chemical Safety of 30 µg/kg bw/day. MGO is also in Group 3 and does not have a specific tolerable daily intake. Finally, while unclassified by the International Agency for Research on Cancer, DA has a notable acceptable daily intake of 900 µg/kg bw/day [245–250].

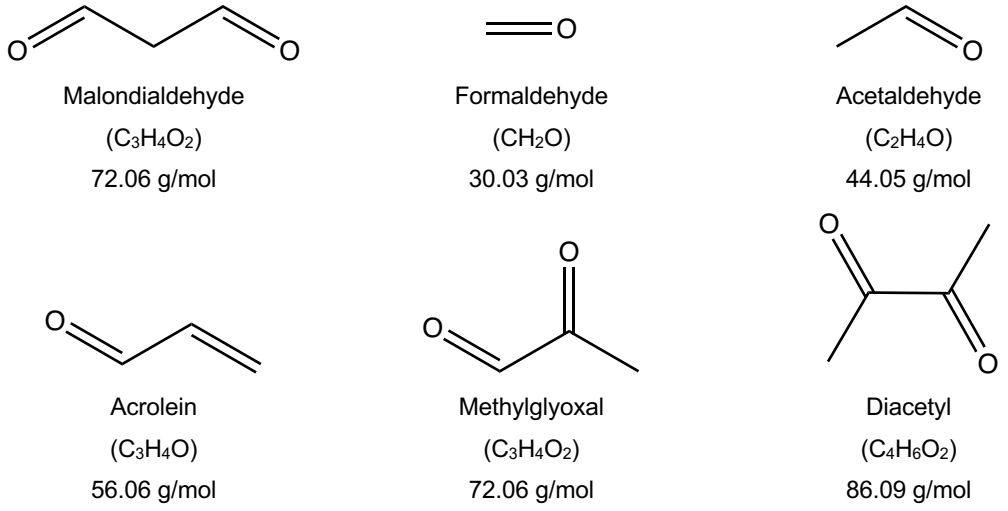


Figure 22 – Structural formula, molecular formula, and molecular weight of target carbonyl compounds.

Given the potential health implications of these contaminants, the need for reliable analytical methods to assess their levels in adult formulas is paramount [245]. Traditional methods for determining lipid peroxidation products in foods, such as the thiobarbituric acid (TBA) test and peroxide value measurement, are widely used due to their simplicity and accessibility [245,251,252]. However, these methods have several limitations: they are non-specific (which can lead to overestimation), time-consuming, and require harsh conditions and large amounts of solvents, which have a notable environmental impact [251,252].To address these limitations, chromatographic methods such as HPLC and GC, paired with various detection systems (UV, Fluorescence Detection (FLD), Flame Ionization Detection, and Mass Spectrometry (MS)), have been developed [245,251–258]. These methods allow for the specific determination of lipid peroxidation products, including aldehydes, ketones, MDA, and dicarbonyl compounds, with higher sensitivity and selectivity than traditional methods [245]. However, to detect specific compounds like advanced glycation end-products and other lipid peroxidation products effectively, chemical derivatization (e.g., with hydrazine, TBA, or OPDA) is often required to enhance instrument response, depending on the detector system [251,255–258]. Microextraction techniques have further advanced the sensitivity and specificity of carbonyl compounds

and VOCs determination by offering a more sustainable approach due to the reduced sample size and waste generation and the reduction (DLLME) or non-use (SPME) of organic solvent [245]. In this context, HS-SPME has been used to determine carbonyl compounds associated with lipid peroxidation in infant formula, milk, and dairy products [245,258,259]. VOC determination without derivatization has also been reported using HS-SPME [245]. DLLME has been applied to measure FCHO, MDA, and ACRL [245,254,260] in beverages. To our knowledge, there is no analytical method for assessing lipid peroxidation products in adult formula products.

In this study, we aim to apply innovative microextraction techniques to determine the concentrations of carbonyl compounds and to monitor VOCs in adult formulas. Specifically, we propose using DLLME for the targeted determination of carbonyl and dicarbonyl compounds as potential markers of lipid peroxidation and HS-SPME for untargeted VOC determination, as it requires no derivatization. Through these methods, we present the first known data on the occurrence of carbonyl compounds associated with oxidative and storage stability in adult formulas, as far as we know. Additionally, we assess the potential risk of exposure for consumers to these contaminants, highlighting the need for ongoing monitoring to warrant the safety and quality of adult formula.

C.1.2 Materials and Methods

C.1.2.1. Chemicals and Reagents

Unless otherwise specified, all chemicals used were high purity (> 98 %). Acetaldehyde (CAS 75-07-0), acetonitrile (CAS 75-05-8), chloroform ($CHCl_3$, 35 %, CAS67-66-3), tetradeuteroacetaldehyde (ACE-d4, CAS 1632-89-9), hexadeuteroacetone (ACO-d6, CAS 666-52-4), diacetyl (CAS 431-03-8), 2,4-dinitrophenylhydrazine (CAS 119-26-6), formaldehyde (CAS 50-00-0), malondialdehyde (CAS 643-12-9), and methylglyoxal (40 % CAS 78-98-8) were all supplied by Merck (Darmstadt, Germany). Acrolein (CAS 107-02-8) was purchased from LGC Standards (London, UK).

SPME fibers with 50/30 µm divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and 65 µm PDMS/DVB were also obtained from Merck. All fibbers were conditioned to ensure optimal performance *per* the manufacturer's instructions before their initial use. Manual sampling was performed using a manual holder sourced from Merck. This study utilized various pieces of laboratory equipment, including a CentromixII-BL Centrifuge from J. P. Selecta (Barcelona, Spain), a Basic 20 pH meter

from Crison Instruments (Barcelona, Spain), a 2510EMTH ultrasonic bath from Branson Ultrasonics (Danbury, CT, USA), and a Reax Top vortex mixer from Instruments GmbH & Co. (Schwalbach, Germany) to conduct all experiments.

C.1.2.2. Sample Selection and Stability Study Design

In this study, 12 commercially available adult nutritional formulas were selected from supermarkets and parapharmacies in Santiago de Compostela, Spain. The formulas were categorized into three groups: high-protein formulations (HP, n = 5), standard formulations (SAF, n = 4), and specialized formulations (SF, n = 3) encompassing an enteral formula for dysphagia and amylase resistance, a carbohydrate module formula, and an enteral powder for administration via tube or orally. All samples were kept in their original packaging before the occurrence study. From these, two unflavoured standards and two unflavoured high-protein adult formulas with similar compositions were selected for the storage stability study. The stability study aimed to evaluate the effects of different storage temperatures on the formulations over 30 days. Unflavoured samples, including high-protein (n = 2) and standard formulation (n = 2), were selected for this study. All samples were transferred into amber vials and the caps were covered with parafilm to prevent contamination. The formulas were stored in their original powdered form and were prepared according to the manufacturer's instructions by dissolving them in drinking water before analysis. Three storage conditions were tested: room temperature, 4 °C, and 60 °C. These conditions were selected to replicate typical domestic storage situations throughout the year. The samples were stored in the dark, using amber vials covered with aluminium foil and placed inside cardboard boxes to minimize light exposure. The four samples were analysed on specified days (0, 1, 7, 14, 21, and 30). In the untargeted VOC determination, focusing on determining VOC changes during storage, two unflavoured samples (also used in the target analysis) from the standard formulation category were selected for HS-SPME-GC-MS analysis after 30 days of storage, as they were the only formulations available in individual packaging of small portions. All analyses were conducted in triplicate to ensure reliable data.

C.1.2.3. Ultrasound-Assisted Dispersive Liquid-Liquid Microextraction

To extract FCHO, MDA, ACE, ACRL, MGO, and DA from the adult formula, a previously developed method to extract MDA, ACRL, and 4-hydroxy-2-nonenal in

beverages was used (Custodio-Mendoza et al., 2022) with modifications [260]. The adult formula was prepared following the manufacturer's instructions: 15 % w/v in drinking water at 40 °C, with gentle stirring until fully dissolved. A 0.5 mL aliquot of the solution was transferred to a 2.5 mL falcon tube, and 1.3 mL of ACN was added. The mixture was vortexed for 1 min to combine the phases, followed by centrifugation for 2 min at 1634 *x g* to precipitate proteins. The supernatant was transferred to a clean tube, and 90 µL of $CHCl_3$ was added. The mixture was homogenized by three cycles of charging and discharging using a Pasteur pipette, then rapidly transferred to a conical tube containing 4 mL of ultrapure water and 0.5 mL of DNPH solution (0.5 g/L in 2 mol/L HCl). The system was incubated in an ultrasonic water bath at 60 °C for 20 min, followed by centrifugation for 2 min at 2146 × *g* to separate the extractant phase. The drop containing the carbonyl-DNPH derivatives was collected with a micro syringe and directly injected into the GC-MS for target analysis.

C.1.2.4. Head-Space Solid-Phase Microextraction

The VOCs were extracted from the adult formula following the method described by Clarke et al., (2019) with modifications [259]. Briefly, the adult formula was prepared as previously outlined. A 2 mL aliquot of the sample solution was placed in a 6 mL vial with a magnetic stirrer and sealed with a septum cap. The sample was equilibrated in a water bath with gentle stirring for 15 min at 40 °C. After the equilibration period, the SPME fibber was exposed, and extraction started for 45 min at the same temperature. Following extraction, the analytes were desorbed at 270 °C for 5 min in the GC-MS injection port.

C.1.2.5. Gas Chromatography-Mass Spectrometry

The targeted and untargeted analyses were performed using a GC–MS system (7890B-5977B, Agilent Technologies, Santa Clara, CA, USA) with a J&W HP-5MS column (30 m × 0.25 mm ID × 0.25 µm, Agilent) and a carrier gas flow rate of 1.5 mL/min. The transfer line was set to 280 °C, connecting the column to an electron impact (EI+) source at 250 °C with 70 eV, and a single quadrupole mass analyser was maintained at 120 °C.

For the targeted analysis, the injector temperature was set to 245 °C using an ultra-inert double taper liner in splitless mode. The temperature program began at 100 °C, increased at 100 °C/min to 230 °C (held for 3.3 min), then ramped at 35 °C/min to a final

temperature of 280 °C, maintained for 2 min. The total analysis time for this targeted method was 8 min. Initially, full scans were performed over a 50–300 m/z range for each hydrazone, allowing the identification of the most intense ions. Single ion monitoring mode was then used with the quantifier and qualifier ions summarized in Table 16.

Table 16 – Retention time, quantifier and qualifier ions for the US-DLLME-GC-MS method.

Analyte	Retention time min	Quantifier ion m/z	Qualifier ions	
			m/z	m/z
MDA	4.03	234	204	207
FCHO	4.03	210	180	211
ACE	4.97	224	225	180
ACE-d4*	4.81	228	227	229
ACO-d6*	5.44	244	243	183
ACRL	5.50	236	189	201
MGO	6.07	252	253	236
DA	6.18	266	267	181

*used as IS.

For the untargeted analysis, the oven temperature started at 35 °C, held for 5 min, and increased at 5 °C/min to 280 °C (held for 10 min), resulting in a total run time of 64 min. The detector was turned off after 60 min. The injector was set to 270 °C using straight ultra-inert liner (5190-4048) with a 0.755 mm inner diameter in splitless mode. Scan acquisition mode covered a 40–400 m/z range. Preliminary compound identification was performed using the NIST Mass Spectral Search Program (Version 2.2) by comparing experimental and reference mass spectra in the NIST/EPA/NIH Mass Spectral Library. Compound identification was based on matching fragmentation patterns and relative ion intensities, with only matches having a probabilistic score above 80 % reported.

C.1.2.6. Analytical Validation

The analytical validation for matrix and analyte extension of the previously published DLLME-GC-MS method was conducted according to the FDA guidelines [261]. The acceptability criteria included several key components. First, the method demonstrated selectivity by providing specific retention times and identifying qualitative and quantitative ions for each analyte, ensuring accurate differentiation between compounds in the matrix. Linearity was assessed using a standard addition method with internal standard (IS) calibration within six concentration levels across a concentration

range of 0.5 to 3 µg/mL for each analyte in triplicate, with strong linear relationships confirmed by high determination coefficients (r^2). The limit of detection (LOD) was calculated as the mean of the blank signal plus 3.3 times the standard deviation of this measurement. Similarly, the LOQ was determined as the mean of the blank signal plus 10 times the standard deviation of the blank. The method's accuracy was evaluated at three concentration levels, with each level tested in quintuplicate, and recovery rates were calculated to assess the closeness of results to the true values. Finally, precision was evaluated by intraday and interday measurements at three concentration levels, each tested in quintuplicate, ensuring consistent results overtime and under different conditions.

C.1.2.7. Statistical Analysis

Statistical analyses were conducted using Excel and Statistica (version 13.3) software. A box-and-whisker plot was used to present the occurrence data, showing the distribution of carbonyl compound concentrations for each sample. This type of plot displays the data's minimum, first-quartile, median, third-quartile, and maximum values. The "box" part represents the interquartile range, which covers the middle 50 % of values. The line inside the box marks the median, indicating the central value of the data set. The "whiskers" extend to the minimum and maximum values, showing the full spread of the data, while any outliers are shown as individual points outside the whiskers. This plot helps to visualize the range, central tendency, and variability in carbonyl compound concentrations across samples. A heatmap was generated to present the storage stability data, visually representing the data trends over time and across different storage conditions. The heatmap illustrated the relative stability of the analytes, with colour gradients indicating the intensity of the responses. Darker shades represented higher concentrations, while lighter shades indicated lower concentrations, allowing for quick identification of significant changes in analyte levels.

C.1.3 Results and Discussion

C.1.3.1. Method Performance – Matrix and Analyte Extension

The US-DLLME-GC-MS method was previously developed in our lab to determine MDA and ACRL in beverages [260]. Herein, we studied the method's applicability to four additional carbonyl compounds (FCHO, ACE, MGO, and DMGO) in adult formula. We used a standard adult formula as a blank sample to ensure the method's suitability for

the simultaneous extraction of these six analytes from the adult formula. First, a kinetic study was performed in triplicate to assess the formation of carbonyl-DNPH derivatives (Figure 23). MDA and ACRL reached equilibrium after 5 min of ultrasound treatment, FCHO after 10 min, while ACE, MGO, and DMGO required 20 min. To ensure all analytes reached equilibrium and were successfully extracted as their corresponding DNPH derivatives, we set the ultrasound incubation time to 20 min. The analytical performance of the method was evaluated under these conditions (Table 17).

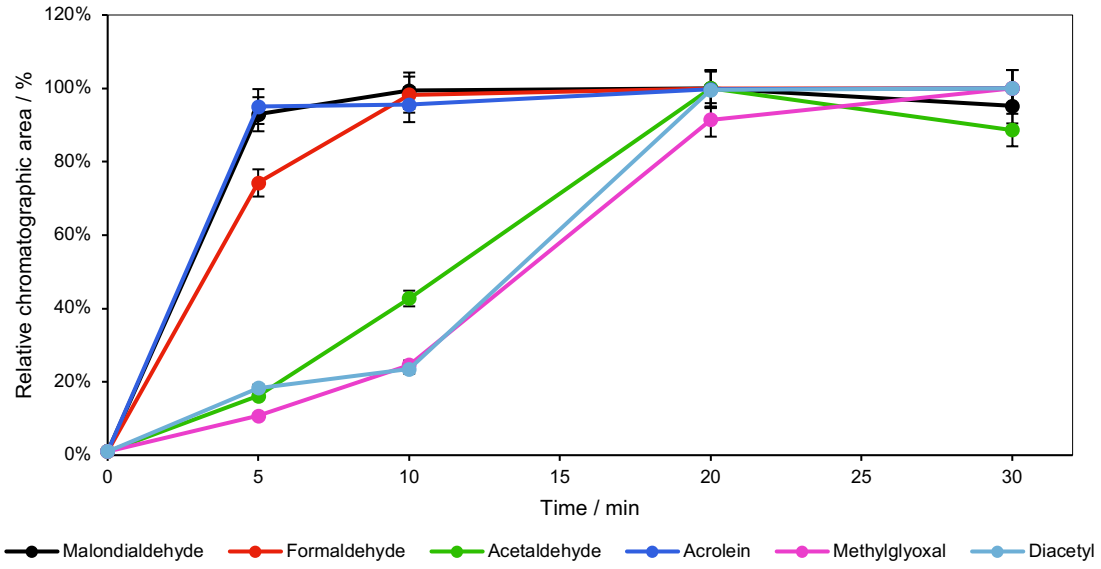


Figure 23 – Kinetic analysis of DNPH derivatization and extraction of carbonyl compounds from adult formula using US-DLLME-GC-MS.

Table 17 – Analytical characteristics of matrix and analyte extension study for the US-DLLME-GC-MS.

Analyte	LOD	LOQ	Slope	Intercept	r^2	Intraday Precision (n = 5) %RSD			Intraday Precision (n = 5) %RSD			Accuracy (n = 5) % Recovery		
	µg/mL	µg/mL	$x10^{-3}$			QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
FCHO	0.022	0.671	24.90	2.3749	0.9997	3.9	2.6	2.9	2.4	1.1	1.5	102.6	98.5	98.5
MDA	0.008	0.195	2.979	0.027	0.9999	4.7	1.9	2.2	4.3	1.7	1.1	100.9	98.5	99.4
ACE	0.089	0.340	3.968	0.0981	0.9999	2.0	5.8	0.4	4.6	0.9	1.2	98.0	99.2	100.0
ACRL	0.017	0.061	1.999	0.0067	0.9998	5.5	4.2	2.7	3.1	3.5	1.5	100.4	99.4	98.6
MGO	0.083	0.229	0.574	0.0499	0.9990	3.4	2.8	6.9	2.4	0.9	3.4	98.0	95.8	93.1
DAI	0.065	0.184	1.552	0.0889	0.9997	1.3	2.1	1.5	1.7	2.2	1.0	97.2	97.2	98.2

n, number of replications; QC1, low-level quality control sample spiked at 0.7 µg/mL; QC2, mid-level quality control sample spiked at 1.0 µg/mL; QC3, high-level quality control sample spiked at 2.0 µg/mL.

The method's specificity is based on specific ions for each analyte and ISs eluting at specific retention times previously described in Section C 1.2.5. Figure 24 confirms the absence of interfering signals in the retention time region for all analytes and the ISs. The LOD ranged from 8 to 89 ng/mL, and the LOQ ranged from 0.061 to 0.671 µg/mL. Standard addition calibrations showed excellent linearity, with determination coefficients of ≥0.9990. The method's precision, assessed at concentrations of 0.7 µg/mL (QC1), 1.0 µg/mL (QC2), and 2.0 µg/mL (QC3), demonstrated good intraday precision with RSD between 1.3 % and 5.8 %, and interday precision with RSD between 0.9 % and 4.6 %. Similarly, method accuracy at the same concentrations showed recoveries ranging from 98.0 % to 102.6 %.

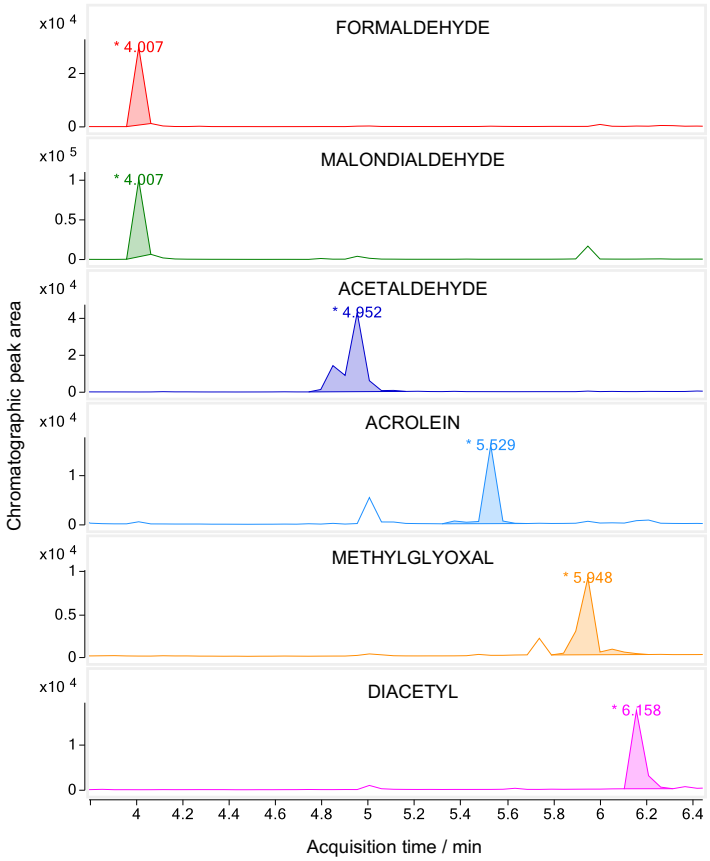


Figure 24 – Chromatogram of standard adult formula spiked with 1 µg/ml of carbonyl compounds.

Table 18 compares the analytical performance of this method with other methods for detecting carbonyl compounds in infant formula, milk, and dairy products. Single HPLC-UV determinations of MDA have been reported, using either the TBA reactive substances test or as a TBA-MDA derivative after liquid–liquid extraction or dilution. Despite being feasible and reproducible, with acceptable RSD values, these methods exhibit poorer linearity and lower recovery rates than those in this study. Similarly, single UV determinations of FCHO in milk have shown higher LOD and LOQ values than those reported here. Single MDA determination in infant formula using HPLC-MS without

derivatization has been reported to have significantly lower detection limits than other HPLC methods. When paired with derivatization, HPLC-MS methods achieve even further reductions in detection limits, as reported for MGO and DA determination in baby food by Kocada˘glı & Gökmen (2014), and in milk and dairy products reported by Zhang et al., 2022 [256,262]. In a previous study, we identified five carbonyl compounds, including MDA, ACRL, MGO, and DA, in infant formula using GDME with OPDA derivatization via HPLC-UV; this method, though accurate and precise, had higher detection limits [257]. Additionally, we previously reported the development of an HS-SPME-GC-MS method for seven carbonyl and dicarbonyl compounds (including FCHO, ACE, MGO, DA, and MDA) as pentafluorophenyl hydrazine (PFPH) derivatives from infant formula, achieving detection limits similar to or slightly lower than those reported here. However, this method had higher RSD values [258].

Overall, while combining derivatization with HPLC-MS produced the lowest detection limits, the extended method reported here achieved similar or lower detection limits compared to previous GC-MS and HPLC-UV methods for milk, dairy products, and infant formula, with more accurate recoveries (closer to 100 %) and comparable precision, proving the suitability of this method for simultaneous monitoring of MDA, FCHO, ACE, ACRL, MGO, and DA in adult formulas.

Table 18 – Analytical methods for MDA, ACRL, and α-DC compounds in infant and adult formula, milk, and dairy products.

Sample	Analytes	Sample preparation	Derivatization	Apparatus	LOD ng/g	LOQ ng/g	r^2	Recovery %	RSD %	Ref
Infant formula	MDA	LLE	TBA	HPLC-UV	NR	NR	0.9827	40-80	<13.7	[251]
Infant formula	MDA	Dilution	TBA reactive substances method	HPLC-UV	9.7	NR	0.9986	70-78	4.6	[252]
Infant formula	MGO	MWAH	-------	HPLC-MS	4.5	18.1	0.996	103	<8.1	[253]
Milk	FCHO	DLLME	ACAC	UV	$1x10^5$	$5x10^5$	NR	91-103	<3.1	[254]
Baby food	MGO, DA	SLE	OPDA	HPLC-MS	2.7-6.0	9.2-20.1	NR	96-103	<5	[255]
Milk and diary	MGO, DA	Protein precipitation	OPDA	HPLC-MS/MS	5-7	18-23	>0.99	85-83	<8.4	[256]
Infant formula	MDA, ACRL, MGO, DA	GDME	OPDA	HPLC-UV	30-300	100-1000	>0.9993	93-105	<4.8	[257]
Infant formula	MDA, FCHO, ACE, MGO, DA	SPME	PFPH	GC-MS	15-50	50-150	>0.9990	84-111	<14.7	[258]
Adult formula	MDA, FCHO, ACE, ACRL, MGO, DA	DLLME	DNPH	GC-MS	8-89	61-671	>0.9990	98-102	<6.9	This work

ACAC – acetylacetone in an ammonium acetate (2.0 % v/v) / MWAH – Microwave-assisted hydrolysis / SLE – Solid-Liquid Extraction / NR – not reported.

C.1.3.2. Occurrence of Carbonyl Compounds in Adult Formula

The validated US-DLLME-GC-MS method was applied to analyse carbonyl compounds in a set of adult formula samples, with results summarized in Table 19.

Table 19 – Occurrence of carbonyl compounds in adult formula.

Adult formula	MDA		FCHO		ACE		ACRL		MGO		DA	
	µg/mL	±SD	µg/mL	±SD	µg/mL	±SD	µg/mL	±SD	µg/mL	±SD	µg/mL	±SD
HP1	2.38	0.39	2.86	0.90	2.16	0.31	2.29	0.73	1.50	0.28	ND	----
HP2	0.98	0.05	0.94	0.03	1.90	0.12	0.24	0.00	ND	-----	ND	----
HP3	ND	----	ND	----	ND	-----	ND	----	ND	-----	ND	-----
HP4	0.53	0.02	1.20	0.50	1.08	0.67	2.39	0.94	1.89	0.77	2.84	0.98
HP5	2.89	0.56	2.60	0.70	ND	-----	2.80	0.17	0.61	0.08	ND	-----
SAF1	2.65	0.82	0.90	0.11	1.63	0.45	1.27	0.70	1.65	0.77	ND	-----
SAF2	1.73	0.54	1.56	0.31	2.91	0.23	1.43	0.35	2.69	0.82	0.97	0.27
SAF3	ND	----	ND	----	ND	-----	ND	----	ND	-----	ND	----
SAF4	ND	-----	ND	----	ND	-----	ND	----	ND	-----	ND	----
SP1	2.09	0.75	1.65	0.75	2.49	0.24	2.49	0.76	2.02	0.52	2.11	0.49
SP2	2.00	0.80	1.48	0.51	1.89	0.53	1.89	0.42	2.46	0.93	1.67	0.96
SP3	ND	----	2.39	0.31	2.53	0.24	2.53	0.56	1.16	0.14	1.88	0.30

SD, standard deviation; SP, special formulated adult formula; ND, non-determined as below limit of quantification.

All target analytes were detected, although not always at quantifiable levels; therefore, a box-and-whisker chart showing only quantifiable samples is presented for clearer comparison (Figure 25). In high-protein adult formulas, 80 % of samples were quantifiable for MDA (0.53–2.89 µg/mL), FCHO (0.94–2.86 µg/mL), and ACRL (0.24–2.80 µg/mL). Additionally, 60 % of samples were quantifiable for ACE (1.8–2.16 µg/mL) and MGO (0.61–1.89 µg/mL), with one sample quantifiable for DA at 2.94 µg/mL. In standard adult formulas, 50 % of samples were quantifiable for MDA (1.73–2.65 µg/mL), FCHO (0.9–1.56 µg/mL), ACE (1.63–2.91 µg/mL), ACRL (1.27–1.43 µg/mL), and MGO (1.65–2.69 µg/mL), with only one sample quantifiable for DA at 0.97 µg/mL. For specialized formulations, only one enteral powder sample, intended for oral or tube administration, was quantifiable for MDA. All analytes in this sample type were quantifiable, with levels ranging from 2.0–2.09 µg/mL for MDA, 1.48–2.39 µg/mL for FCHO, 0.79–1.16 µg/mL for ACE, 1.89–2.49 µg/mL for ACRL,1.16–2.46 µg/mL for MGO, and 1.67–2.11 µg/mL for DA.

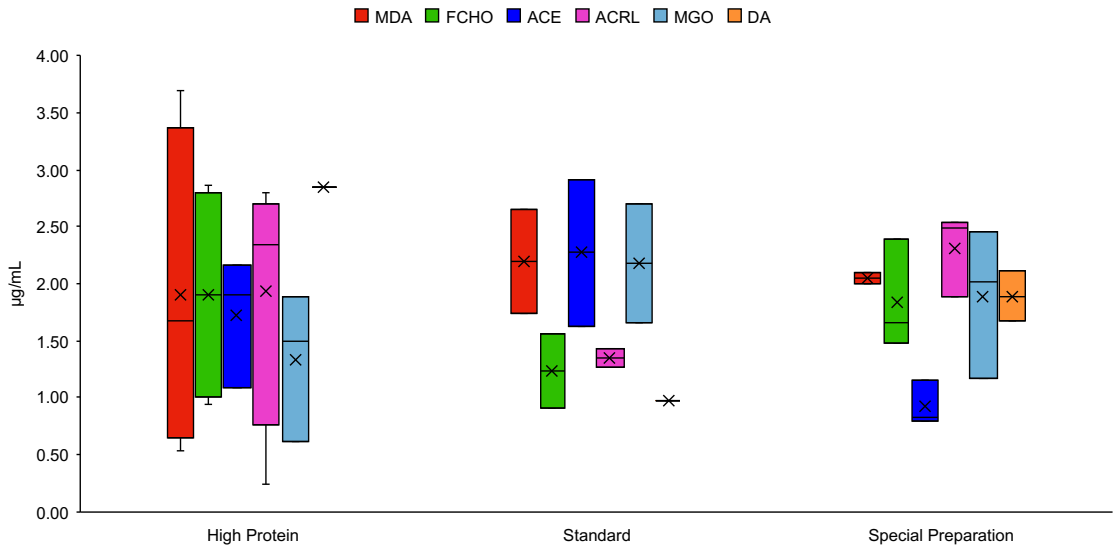


Figure 25 – Box-and-Whisker plot showing the occurrence of carbonyl compounds in adult formula samples.

To our knowledge, no studies have reported the occurrence levels of these compounds, specifically in adult formula. However, Kang et al. (2010) conducted short- and long-term trials to assess the effects of commercial enteral nutritional supports on the nutrition and health of stroke patients. They observed that MDA was significantly elevated in chronic stroke patients compared to healthy individuals after TBA derivatization and HPLC-UV [263].

Regarding similar foods, Bessaire et al. (2018) reported similar FCHO levels in milk powders after DNPH derivatization and HPLC-tandem mass spectrometry [264]. Custodio-Mendoza et al., (2024) observed lower levels of MDA, FCHO, and ACE in both powdered and liquid starter and follow-up infant formula after pentafluorophenyl hydrazine derivatization during HS-SPME extraction and GC-MS determination [256]. Similar ACRL, MGO, and DA levels were found in powdered starter formulas and ACRL levels in follow-up formulas after OPDA derivatization during GDME extraction and HPLC-UV determination [258]. Similar MDA content in infant formula was reported by Pozzo et al. using TBA derivatization and HPLC-UV-FLD [265] and Cesa using the TBA reactive substances test [266]. Akıllıo˘glu et al. with their microwave-assisted hydrolysis and HPLC-MS determination [253], as well as Kocada˘glı et al. [262], reported comparable MGO results in the analysis of infant formulas, supporting these findings

These findings suggest that MDA, FCHO, ACRL, ACE, MGO, and DA concentrations vary significantly across adult formula samples. The data suggest that high-protein and specialized formulations may be more susceptible to carbonyl compound formation than standard formulas. This variability highlights the importance of

monitoring carbonyl compound levels in these products as potential indicators of oxidation which could affect product quality and safety.

C.1.3.3. Temperature-Dependent Variations of Carbonyl Compound Levels in Adult Formulas During Storage

Four samples were selected in the target stability study using the US-DLLME-GC-MS method. They included two standard adult formulas and two high-protein adult formulas from the same brand, differing only in protein content. Carbonyl compounds were detected in all samples, though quantification was only possible in HP samples at all time periods and in SAF at later stages. Results are summarized in Table 20.

In HP formulas, MDA levels increased over time under different storage conditions. At 4 °C, MDA remained relatively stable, detectable only in one HP sample, showing a slight increase at the end of 30 days. In the other HP sample, MDA became quantifiable by day 30. At room temperature, MDA accumulated more, reaching 2.01 µg/mL and 0.37 µg/mL after 30 days. Storage at 60 °C showed a similar trend, with MDA reaching comparable levels. MGO also showed accumulation over time, becoming detectable in HP samples after 7 days at 4 °C and increasing to 0.64 µg/mL and 0.98 µg/mL after 30 days. MGO levels rose further at higher temperatures, reaching 1.52–1.98 µg/mL after 30 days at room temperature and 2.54–2.78 µg/mL after 30 days at 60 °C. FCHO accumulated more gradually at 4 °C, becoming quantifiable only after 30 days at 0.5 µg/mL in one HP sample, while the other sample showed a higher range (0.94–1.93 µg/mL). FCHO accumulation intensified with higher storage temperatures, reaching up to 2.73 µg/mL at 60 °C after 30 days. In contrast, ACE and ACRL showed only slight and relatively stable increases across all temperatures.

Table 20 – Variations of carbonyl content in adult formulas during 1 month of storage at different temperatures.

	Analyte	4 ºC					Room Temperature					60 ºC				
		1	7	14	21	30	1	7	14	21	30	1	7	14	21	30
HP2	MDA	1.22 ± 0.41	1.19 ± 0.99	1.54 ± 0.83	1.49 ± 0.95	1.41 ± 0.40	0.86 ± 0.09	0.85 ± 0.06	1.17 ± 0.10	1.99 ± 0.04	2.01 ± 0.04	0.97 ± 0.08	1.01 ± 0.04	1.91 ± 0.06	1.91 ± 0.16	2.23 ± 0.16
	FCHO	0.95 ± 0.45	1.14 ± 0.63	1.43 ± 0.33	1.64 ± 0.13	1.93 ± 0.22	0.94 ± 0.04	1.24 ± 0.05	1.51 ± 0.03	1.93 ± 0.09	2.03 ± 0.04	0.95 ± 0.04	1.24 ± 0.04	1.74 ± 0.09	2.13 ± 0.05	2.73 ± 0.05
	ACE	1.92 ± 0.27	2.06 ± 0.03	2.08 ± 0.25	2.53 ± 0.60	2.37 ± 0.65	2.03 ± 0.23	2.07 ± 0.20	2.19 ± 0.59	2.62 ± 0.40	2.83 ± 0.03	1.96 ± 0.07	2.00 ± 0.09	2.46 ± 0.04	2.47 ± 0.16	2.94 ± 0.23
	ACRL	0.24 ± 0.53	0.24 ± 0.05	0.38 ± 0.02	0.43 ± 0.01	0.44 ± 0.01	0.26 ± 0.06	0.22 ± 0.06	0.39 ± 0.06	0.40 ± 0.03	0.45 ± 0.04	0.24 ± 0.08	0.34 ± 0.04	0.33 ± 0.06	0.42 ± 0.06	0.56 ± 0.08
	MGO	ND	0.31 ± 0.08	0.48 ± 0.05	0.57 ± 0.02	0.64 ± 0.03	ND	0.32 ± 0.04	1.22 ± 0.03	1.75 ± 0.06	1.98 ± 0.01	0.23 ± 0.05	1.49 ± 0.01	1.60 ± 0.03	2.08 ± 0.09	2.78 ± 0.06
	DA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HP3	MDA	ND	ND	ND	ND	0.28 ± 0.04	ND	ND	ND	ND	0.37 ± 0.03	ND	ND	0.30 ± 0.04	0.30 ± 0.06	0.40 ± 0.02
	FCHO	ND	ND	ND	ND	0.50 ± 0.08	0.77 ± 0.01	0.76 ± 0.02	0.80 ± 0.04	0.83 ± 0.08	0.92 ± 0.01	0.84 ± 0.07	0.88 ± 0.09	0.92 ± 0.09	0.96 ± 0.05	0.97 ± 0.07
	ACE	0.99 ± 0.01	1.44 ± 0.25	1.32 ± 0.27	1.36 ± 0.15	1.38 ± 0.17	1.13 ± 0.06	1.51 ± 0.12	1.81 ± 0.10	1.95 ± 0.33	2.39 ± 0.25	1.03 ± 0.31	1.65 ± 0.37	1.91 ± 0.28	2.15 ± 0.25	2.57 ± 0.17
	ACRL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MGO	ND	0.35 ± 0.02	0.67 ± 0.17	0.71 ± 0.15	0.98 ± 0.10	0.65 ± 0.03	1.05 ± 0.25	1.19 ± 0.13	1.23 ± 0.44	1.52 ± 0.27	0.82 ± 0.04	1.01 ± 0.04	1.43 ± 0.05	2.34 ± 0.09	2.54 ± 0.07
	DA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, non-determined as it was below the limit of quantification.

Table 19 – Continue.

	Analyte	4 ºC					Room Temperature					60 ºC				
		1	7	14	21	30	1	7	14	21	30	1	7	14	21	30
SAF3	MDA	ND	ND	ND	ND	0.28 ± 0.06	ND	ND	0.28 ± 0.04	0.48 ± 0.05	0.58 ± 0.03	ND	ND	0.36 ± 0.01	0.57 ± 0.03	0.88 ± 0.09
	FCHO	ND	ND	ND	ND	0.71 ± 0.02	ND	ND	1.26 ± 0.01	0.13 ± 0.01	1.79 ± 0.09	ND	0.93 ± 0.09	1.41 ± 0.05	1.55 ± 0.05	1.79 ± 0.02
	ACE	ND	ND	ND	ND	0.37 ± 0.08	ND	ND	ND	ND	0.57 ± 0.08	ND	ND	ND	0.55 ± 0.05	0.62 ± 0.01
	ACRL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MGO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SAF4	MDA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.32 ±0.02
	FCHO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.62 ±0.02
	ACE	ND	ND	1.12 ±0.01	1.26 ±0.07	1.38 ±0.06	ND	ND	1.81 ±0.23	1.85 ±0.27	1.94 ±0.44	ND	ND	1.91 ±0.09	2.15 ±0.27	2.27 ±0.57
	ACRL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MGO	ND	ND	ND	0.25 ±0.01	0.71 ±0.08	ND	ND	ND	0.88 ±0.02	1.23 ±0.05	ND	ND	1.43 ±0.25	1.54 ±0.43	1.94 ±0.13
	DA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

In SAF samples, most compounds were undetectable until 7 days at both 4 °C and room temperature. MDA was below the limit of quantification in one SAF sample throughout the study, and ACRL and DA were undetectable in all SAF samples across temperatures. At 4 °C, only ACE was quantifiable in one sample after 14 days, rising to 0.37–1.38 µg/mL after 30 days. MGO was quantifiable in one SAF sample after 21 and 30 days at 0.25 and 0.71 µg/mL, respectively. In the other SAF sample, MDA, FCHO, and ACE were quantifiable only after 30 days, with concentrations of 0.28, 0.71, and 0.37 µg/mL, respectively. MDA and FCHO became quantifiable at room temperature in one SAF sample after 14 days, reaching 0.58 and 1.79 µg/mL by day 30. ACE was quantifiable in both SAF samples after 30 days, with concentrations of 0.57–1.94 µg/mL, while MGO reached 1.23 µg/mL in one sample after 21 days. At 60 °C, MDA became quantifiable after 14 days, reaching 0.32–0.88 µg/mL after 30 days. FCHO was quantifiable in one sample after 7 days, accumulating from 0.93 to 1.79 µg/mL by day 30. ACE became quantifiable in both samples after 14 and 21 days, reaching 0.62–2.27 µg/mL after 30 days, while MGO was only quantifiable in one sample after 14 days, reaching 1.43–1.94 µg/mL.

Although no data is available on the variations in carbonyl content in adult formula during one month of storage, our findings are consistent with those reported by other researchers in infant formula, milk, and dairy products. Bessaire et al. (2018) noted temperature-related differences in FCHO levels in milk powders, finding that FCHO levels were lower at room temperature than at 60 °C [264]. Cesa et al., (2015) reported a significant increase in MDA content in infant formula stored for two weeks at 55 °C [267]. Jia et al. (2018) found significant differences in content of advanced glycation end products, such as MGO and DA, in milk-based infant formulas stored at 50 °C [268]. Liu and Lial so observed similar variations in MGO after 60 days of storage [269]. Notably, Cheng et al. reported that even moderate increases in temperature can significantly contribute to the formation of advanced glycation end-products, reaching detectable levels in infant formula milk powders, consistent with our results [270].

These findings suggest carbonyl compounds accumulate more over time in HP than in SAF, particularly at higher storage temperatures. The increase of MDA, MGO, and FCHO levels in HP formula indicates that higher protein content may promote oxidative reactions during storage, as reported in other foods [271]. Elevated temperatures further accelerate this process, with the most significant accumulation occurring at 60 °C. This implies that protein content and storage conditions (temperature and duration) are critical factors in adult formulas' stability and potential degradation.

C.1.3.4. Temperature-Dependent Variations of VOC Levels in Adult Formulas During Storage

In the untargeted stability study using HS-SPME-GC-MS, identification was based on comparing the mass spectrometry (MS) patterns with those in the NIST library. The results are summarized in Table 21 and Table 22, and heatmaps were created using the chromatographic peak areas obtained from a set of new SAF samples. These samples were stored for 30 days at 4 °C, room temperature, and 60 °C to facilitate comparison, as quantification was not possible (Figure 26). A total of 40 compounds were observed, including 11 aldehydes, 10 ketones, 9 alcohols, and 2 esters, among others. Notably, hexanal, 2-heptanone, heptanal, 2-heptenal, 2-octenal, nonanal, and 2-hydroxybenzaldehyde were present in both new SAF samples. In contrast, pentyl acetate and 1-octen-3-ol were found in only one sample, while benzaldehyde, 2-pentylfuran, and octanal were found in the other sample.

Table 21 – Volatile compounds identified in adult formulas.

Compound	Retention Time (min)	Molecular Weight (g/mol)	Molecular Formula	CAS Number
Aldehydes				
Hexanal	3.961	100.16	$C_6H_{12}O$	66-25-1
2-Ethylbutenal	4.911	98.15	$C_6H_{10}O$	8/1/4786
Heptanal	7.413	114.19	$C_7H_{14}O$	111-71-7
Benzaldehyde	9.670	106.12	C_7H_6O	100-52-7
Octanal	11.507	128.21	$C_8H_{16}O$	124-13-0
2-Heptenal	9.590	110.18	$C_7H_{12}O$	18829-55-5
trans-2-Octenal	13.830	126.2	$C_8H_{14}O$	2548-87-0
Nonanal	15.634	142.24	$C_9H_{18}O$	124-19-6
2-Hydroxybenzaldehyde	16.292	122.12	$C_7H_6O_2$	90-02-8
Decanal	19.403	156.26	$C_{10}H_{20}O$	112-31-2
2-Undecenal	24.853	168.29	$C_{11}H_{20}O$	2463-77-6

Table 22 – Volatile compounds identified in adult formula.

Compound	Retention Time (min)	Molecular Weight (g/mol)	Molecular Formula	CAS Number
Ketones				
2-Heptanone	7.023	114.19	$C_7H_{14}O$	110-43-0
6-Methyl-5-hepten-2-one	10.874	126.2	$C_8H_{14}O$	110-93-0
3-Octen-2-one	13.010	126.2	$C_8H_{14}O$	1669-45-8
2-Nonanone	15.146	128.21	$C_9H_{18}O$	821-55-6
3,5-Octadien-2-one	15.138	124.18	$C_8H_{12}O$	3/5/4313
3-Nonen-2-one	16.949	126.2	$C_9H_{16}O$	18829-56-6
Cyclodecanone	21.515	154.25	$C_{10}H_{18}O$	1502-06-3
6-Undecanone	21.767	170.29	$C_{11}H_{22}O$	13161-21-6
2-Decanone	18.940	142.24	$C_{10}H_{20}O$	693-54-9
2-Undecanone	22.522	156.26	$C_{11}H_{22}O$	112-12-9
Alcohols				
1-Pentanol	4.676	88.15	$C_5H_{12}O$	71-41-0
1-Hexanol	6.138	102.17	$C_6H_{14}O$	111-27-3
1-Heptanol	10.272	116.2	$C_7H_{16}O$	111-70-6
1-Octen-3-ol	10.638	128.21	$C_8H_{16}O$	3391-86-4
trans-2-Undecen-1-ol	13.335	170.29	$C_{11}H_{22}O$	18409-17-7
1-Octanol	14.261	130.23	$C_8H_{18}O$	111-87-5
1-Decanol	16.568	158.28	$C_{10}H_{22}O$	112-30-1
1-Octanol, 2-butyl-	18.192	186.33	$C_{12}H_{26}O$	2/8/3913
2-Butyl-2,7-octadien-1-ol	25.186	154.25	$C_{12}H_{22}O$	14367-88-9
Esters				
Pentyl Acetate	7.917	130.18	$C_7H_{14}O_2$	628-63-7
Hexanoic acid, pentyl ester	22.351	186.31	$C_{11}H_{22}O_2$	818-29-3
Organic Acids				
17-Octadecynoic acid	17.997	280.46	$C_{18}H_{32}O_2$	59336-89-1
Others				
γ-Octalactone	21.271	142.2	$C_8H_{14}O_2$	104-50-7
Oxime-, methoxy-phenyl	8.347	137.15	C_8H_9NO	100-64-1
Cycloheptene, 1,2-dimethyl	12.587	110.19	C_9H_{16}	1192-37-6
2-Pentylfuran	11.077	138.21	$C_9H_{14}O$	3777-69-3
2-Methyldecalin	17.136	152.27	$C_{11}H_20$	32918-38-6

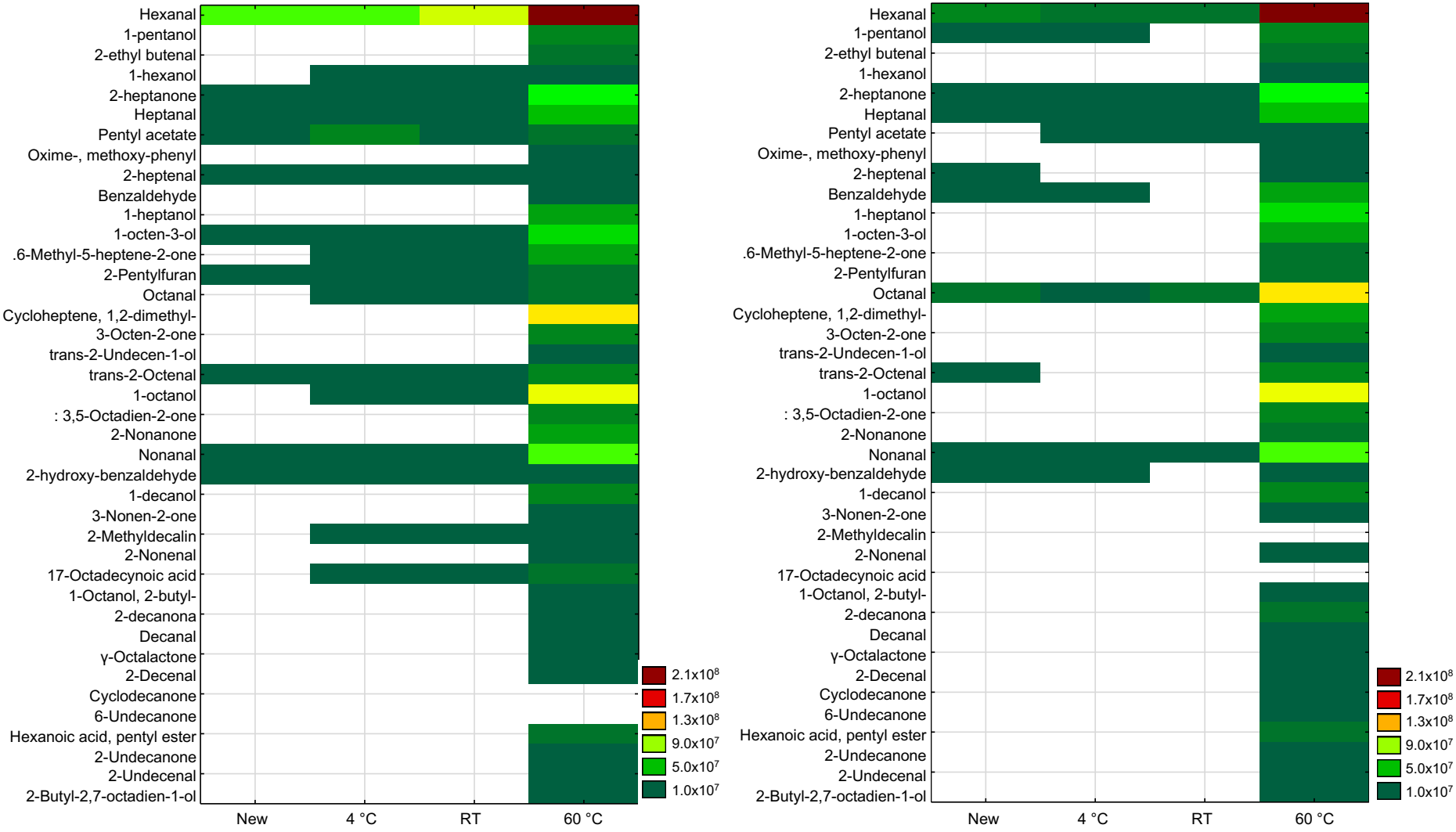


Figure 26 – Heatmap of VOCs identified in adult formula after 1 month storage. Standard adult formula 3 (**left**), standard adult formula 4 (**right**).

Hexanal appears stable at 4 °C, with a slight increase noted in one sample stored at room temperature. However, both samples showed a significant increase in hexanal levels after 30 days at 60 °C. The other compounds in the fresh samples seem stable at both 4 °C and room temperature. In contrast, most compounds exhibited some increase when stored at 60 °C, except for nonanal and 2-hydroxybenzaldehyde, which remained stable at all temperatures. Moreover, compounds such as 1-hexanol, octanal, 1-octanol, 2-methyldecaline, and 17-octadecenoic acid appeared after 30 days of storage at all studied temperatures. Other compounds, including 1-pentanol, 1-heptanol, 2-nonanone, 1-decanol,1-octanol, 2-decanone, decanal, γ-octalactone, 2-decenal, cyclodecanone, 6-undecanone, hexanoic acid pentyl ester, 2-undecanone, 2-undecenal, and 2-butyl-2,7-octadien-1-ol, were only present after 30 days of storage at 60 °C.

Similar VOCs, including aldehydes, ketones, alcohols, and volatile acids, were reported by Jia et al. [268] in their analysis of milk-based infant formulas, as well as by Hausneret al., (2009) in their characterization of infant formulas and breast milk [272]. Li, Zhang, and Wang also noted the formation of aldehydes and ketones in milk powders stored at various temperatures, which aligns with our findings [273]. Li et al., noticed the presence of aldehydes and ketones that influence the sensory properties of infant formulas [237]. They also found that long-term storage (up to one year) at room temperature leads to significant variations in volatile compounds, similar to our observations, with aldehydes and ketones being the most prevalent, significantly impacting the sensory attributes of infant formulas [237].

C.1.3.5. Risk of Exposure Assessment Based on Adult Formula Consumption

In this study, we calculated the estimated consumption of adult formula to assess potential exposure to contaminants. The analysis accounted for differences in caloric needs between males and females to avoid gender bias and individual activity levels, considering the role of adult formula as a meal replacement and the highest carbonyl content identified in the occurrence study [274]. Based on average daily caloric requirements for males and females by activity level (Table 23) and noting that 100 mL of adult formula provides between 100–479 kcal when prepared according to the manufacturer's instructions, we summarized the average daily intake for males weighing 70–90 kg and females weighing 55–75 kg in Table 24, expressed as µg/kg of body weight *per* day. We compared the average daily intakes with the tolerable or acceptable daily intakes for carbonyl compounds (see section C 1.1.) [275].

Table 23 – Average daily caloric request.

Level of Activity	Male		Female	
Low	2200	2400	1800	2000
Moderate	2400	2800	2000	2200
High	2800	3000	2200	2400

Table 24 – Average daily intake.

Level of physical activity	MDA µg / Kg bw /day		FCHO µg / Kg bw /day		ACE µg / Kg bw /day		ACRL µg / Kg bw /day		MGO [a] µg / Kg bw /day		DA µg / Kg bw /day	
	M	F	M	F	M	F	M	F	M	F	M	F
Low	53.5*	55.3*	52.9	69.8	53.8	55.7	51.8*	53.6*	49.8	51.5	52.5	67.7
Moderate	58.9*	61.4*	58.3	60.8	59.3	61.8	57.1*	59.5*	54.9	57.1	57.9	60.3
High	67.9*	67.5*	67.2	66.8	68.3	67.9	65.7*	65.4*	63.2	62.8	66.7	66.3

M, male; F, female; * above tolerable daily intake (or analog exposure level); [a] compare as to GO equivalent as *per* absence of exposure level.

The risk of exposure assessment indicates that, when consuming high-protein adult formula as a meal replacement, adult women with low physical activity may experience slightly higher exposure to FCHO and DA. In contrast, for individuals with moderate to high physical activity levels, exposure to these contaminants is similar for both males and females. While exposure to most of the targeted compounds across the studied weight range and activity levels remains below the tolerable daily intake (or equivalent exposure level), both males and females would be exposed to levels of MDA twice the toxicological threshold of concern and approximately eight times the tolerable daily intake for ACRL.

C.1.4 Conclusions

This study highlights the need for a reliable analytical method to assess lipid peroxidation products in adult formula, a gap not addressed in prior research. By adopting a previously established method for detecting MDA and ACRL in beverages, we success fully extended it to determine six key analytes—MDA, ACRL, FCHO, ACE, MGO, and DA—in adult formulas. The method showed excellent specificity, precision, and accuracy, achieving low limits of detection and quantification.

To our knowledge, this is the first occurrence study of carbonyl compounds in adult formulas. Remarkably, high-protein formulas showed the highest detectability, with 80 % of samples quantifiable for MDA, FCHO, and ACRL and 60 % quantifiable for ACE and MGO. Standard formulas had a 50 % quantification rate for most compounds, while only

one special formulation sample, intended for oral or tube feeding, had quantifiable levels of all analytes.

The analysis of various adult formula samples revealed significant variations in the levels of carbonyl compounds when stored at different temperatures using HS-SPME-GC-MS. High-protein adult formulas consistently exhibited higher concentrations of these compounds than standard formulas. This suggests that higher protein content may enhance the formation of carbonyl compounds during storage, particularly under elevated temperatures. These findings suggest that the protein content and storage conditions—such as temperature and duration—are critical factors influencing the stability and degradation of these products.

The untargeted determination of VOCs by HS-SPME-GC-MS identified a diverse range of compounds, including various aldehydes, ketones, alcohols, and volatile acids. The results indicated that many VOCs accumulate over time, particularly in high-protein adult formulas stored at elevated temperatures. Notably, hexanal, 2-heptanone, and other compounds showed significant increases, suggesting that storage conditions critically affect the stability of these products. The presence of these VOCs, many of which are associated with sensory attributes, emphasizes the importance of understanding their impact on product quality. The findings also suggest that monitoring VOC levels can serve as an indicator of oxidation processes in adult formulas.

Our findings suggest that, once opened, adult formula should be stored in its original packaging, protected from light and moisture, and kept away from heat sources such as ovens or central heating. Formulas with higher protein content should ideally be stored in a refrigerator, particularly during the summer or in locations where the room temperature exceeds 25 °C.

Furthermore, we assessed the potential risk of exposure to these contaminants based on the estimated consumption of adult formula. Our results indicate that males and females, across all activity levels, may exceed safety thresholds for MDA and ACRL when consuming high-protein formulas. This underscores the importance of continuously monitoring carbonyl compounds in adult formulas, as high levels may compromise product quality and safety.

Chapter C 2

Green Solvents in Dispersive Liquid-Liquid Microextraction for the Determination of Carbonyl Compounds in Coffee Extracts

Abstract

This chapter presents a greener approach for ultrasound-assisted (UA) dispersive liquid-liquid microextraction (DLLME) of carbonyl compounds from coffee samples, before GC–MS determination. This work aims to substitute the solvents used in the traditional DLLME procedures with greener alternatives and to decrease the volume of solvents used. Low-density solvents, 1-octanol and isooctane, were evaluated as the extraction solvent. Optimization of critical experimental parameters was conducted in two stages: an asymmetrical screening design $2^3 3^1$ // 8, followed by a Doehlert experimental design. In the first experimental design 4 parameters were optimized: the volume of aqueous phase volume (1.5 mL), the concentration of the derivatization reagent solution pentafluorophenylhydrazine (1.12 g/L) and the volume and type of extraction solvent (60 µL of isooctane). In the second experimental design, 15 min of derivatization at 50 °C were selected as optimized conditions. The enrichment factor associated with the DLLME procedure enabled the efficient extraction of nine carbonyl compounds (acetaldehyde, acrolein, benzaldehyde, diacetyl, formaldehyde, furfural, glyoxal, malondialdehyde, and methylglyoxal) from coffee samples. The method demonstrated strong analytical performance, with figures of merit including $r^2 \geq 0.9990$, limits of detection between 289 and 436 µg/L, intraday, and interday precisions < 9.5 %. Recovery values for all nine carbonyl compounds ranged from 90.0 to 110.0 %. The greenness of the developed methodology was assessed using the AGREEprep tool, yielding a score of 0.59. Acetaldehyde, benzaldehyde and furfural were quantified in most coffee samples analysed, with no significant differences observed in carbonyl compounds composition.

C 2.1 Introduction

Coffee is one of the most consumed beverages worldwide, appreciated for its unique flavor, aroma and beneficial health effects [276]. Its complex composition contains >1000 different compounds, which contribute for the taste, smell and overall quality of the final product [277]. This composition varies depending on the coffee variety, origin, roasting degree, and decaffeination, among other factors [278].

Among the wide range of chemical compounds found in coffee, carbonyl compounds are particularly significant due to their role as key markers of lipid oxidation [279]. Previous studies have highlighted the negative impact of lipid oxidation on coffee during storage, leading to the development of rancid odors, decrease of product quality and formation of secondary oxidation products harmful to the human health [280,281]. Compounds such as FCHO, ACRL and ACE, which are among the oxidation products, are classified by the International Agency for Research on Cancer as carcinogenic or probably carcinogenic to humans. [282]. The importance of quantifying these and other carbonyl compounds led to the development of numerous methodologies with different sample preparation techniques. For instance, Daglia et al. [211] achieved the extraction of α-dicarbonyl compounds from coffee by introducing solid phase extraction (SPE) to remove interfering compounds, followed by HPLC-DAD analysis of the derivatized analytes. Cordeiro et al. [77] proposed a methodology based on GDME followed by HPLC-DAD-MS/MS for the extraction and determination of 27 carbonyl compounds. Santos et al. [101] developed an innovative extraction technique, based on fan assisted air circulation, followed by HPLC-UV, for the extraction of 19 carbonyl compounds. Other published works also used dispersive liquid-liquid microextraction (DLLME) based methodologies to extract these compounds from coffee samples, such as Galuch et al. [96], for the determination of acrylamide, and, more recently, Custodio-Mendoza et al. [260], for the determination of MDA, ACRL and 4-hydroxy-2-nonenal.

Dispersive liquid-liquid microextraction (DLLME) is a widely used sample preparation technique that, despite its simplicity and efficiency, typically involves hazardous solvents in reduced amounts [103]. In a simplified version, DLLME is a ternary solvent system consisting of a majoritarian aqueous phase (containing the analytes), a miniaturized extraction solvent (immiscible with water), and a dispersive solvent (miscible in both phases). Micro-emulsification occurs upon rapidly adding the dispersive and extraction solvents to the aqueous phase, resulting in an enhanced contact surface between the extraction solvent and the aqueous matrix [283]. Phase separation is achieved through centrifugation, with the extraction solvent forming a droplet at the

bottom of the conical tube containing the target analytes extracted from the aqueous matrix. The extraction and pre-concentration of the target analytes, present in the aqueous phase, is achieved through DLLME [104]. This technique is favored for its rapidity, simplicity, high enrichment factor, and efficiency [284]. However, toxic solvents, such as chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2), are often used in conventional DLLME methods [104].

Recent efforts have focused on developing greener DLLME approaches in response to environmental and safety concerns. With green chemistry rapidly becoming the forefront of scientific innovation, driven by the growing demand for sustainable and environmentally friendly solutions, there is an overwhelming need to develop greener alternatives for sample preparation. The principles of green chemistry emphasize the minimization of hazardous substances, reduction of waste, and optimization of energy efficiency [285]. Green chemistry encourages the development of innovative, sustainable methodologies and incorporation of environmentally friendly practices into existing ones [81,286].

Efforts to align DLLME technique with these principles include the use of less toxic extraction solvents, such as ionic liquids [287], and low-density solvents [107], as well as the integration of other sample preparation steps to reduce overall time and reagent consumption [109]. A novel advancement in this field was introduced in 2013 [288], comprising ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) and combining the analytes derivatization and extraction into a single step. Derivatization is a widely employed additional step in sample preparation, particularly when using chromatographic methods involving mass spectrometry (MS), as it enhances analyte detectability by forming high molecular weight and stable compounds that significantly improve the precision and sensitivity of their identification by MS [289,290]. UAE is an established technique for the extraction of organic compounds from solid samples [291] and has seen increasing application in recent years for liquid samples [292]. One critical phenomenon is the formation and collapse of cavitation bubbles, which occurs continuously as the medium is exposed to ultrasounds. This process enhances solvent penetration into the sample matrix, increasing the mass transfer of analytes to the solvent [293]. UAE, when combined with the derivatization step, increases the kinetic energy of the reaction, reducing the derivatization time and the overall sample preparation time [294]. Despite the efficiency demonstrated by these DLLME methodologies, one main drawback, in terms of green chemistry principles, is the use of toxic solvents [295] such as ACN and chloroform ($CHCl_3$) [84].

The present study evaluates low-density solvents, 1-octanol and isooctane, as greener alternatives to $CHCl_3$ as an extraction solvent, and EtOH, a more environmentally friendly solvent [296], as a substitute to ACN as a dispersive solvent. Furthermore, ortho-phosphoric acid (H_3PO_4) and hydrochloric acid (HCl) were studied, in the preparation of the derivatization reagent solution, previous to the optimization studies. Reduced volumes of aqueous and organic phases were also studied, which aligns with green chemistry principles. Validation tests of the methodology were performed according to Food and Drug Administration (FDA) guidelines [261,297]. A greenness assessment was performed, comparing recently published DLLME-based methodologies for extracting carbonyl compounds, especially those targeted in the present work, in food matrices, with the developed methodology and by the AGREEprep software. Thirteen coffee samples were analyzed using the developed methodology and the results were discussed.

C 2.2 Materials and Methods

C 2.2.1 Reagents

Ethanol (CAS. 64-17-5, HPLC grade) was obtained from Scharlab (Quezon City, Philippines). 1-Octanol (CAS. 111-87-5, > 99 %), isooctane (CAS. 540-84-1, for gas chromatography) and pentafluorophenylhydrazine (CAS. 828-73-9, 97 %) were obtained from Merck (Darmstadt, Germany). Ortho-phosphoric acid (CAS. 7664-38-2, 85 % w/w) and hydrochloric acid (CAS. 7647-01-0, 32 % w/w) were obtained from VWR Chemicals (Pennsylvania, USA). Ultrapure water was obtained from a Millipore purification system (Millipore, Billerica, MA, USA).

Analytical standards used, acetaldehyde (CAS. 75-07-0, ≥ 99.5 %), benzaldehyde (PhCHO, CAS. 100-52-7, ≥ 99.5 %), diacetyl (CAS. 431-03-8, ≥ 99 %), deuterated acetaldehyde (CAS. 1632-89-9, ≥ 99 atom %), formaldehyde (CAS. 50-00-0, 37 %), furfural (FUR, CAS. 98-01-1, ≥ 98.5 %), glyoxal (CAS. 107-22-2, 40 %), malondialdehyde tetrabutylammonium salt (CAS. 100683-54-3, ≥ 97 %) and methylglyoxal (CAS. 78-98-8, 40 %), all from Merck. Acrolein (107-02-8, 5000 µg/mL in MEOH) was obtained from Dr. Ehrenstorfer (Augsburg, Germany).

The derivatization reagent PFPH solutions were prepared at 3 concentrations (2.8 g/L, 5.6 g/L and 11.2 g/L), using either HCl (2.0 mol/dm^3) or H_3PO_4, (10.4 mol/dm^3). These solutions were freshly prepared at the begin of each working day.

Individual standard stock solutions were prepared in MeOH (CAS. 67-56-1, hypergrade for LC-MS), obtained from Merck, and stored at 4 ºC. Working and IS, ACE-d4, solutions were freshly prepared with ultrapure water to the desired concentrations.

C 2.2.2 Coffee Samples

Thirteen roasted ground coffee samples, purchased at a local market in Santiago de Compostela, Spain, were analyzed. The samples were divided in 4 types of coffee: 6 samples of regular coffee (RC); 5 samples of coffee mixture (regular and torrefacto (sugar-roasted) coffee) (MC); 1 sample of regular decaffeinated coffee (RDC); and 1 sample of decaffeinated coffee mixture (MDC).

Samples were prepared for analysis by adding 0.15 g of ground coffee beans (accurately weighted) to 25.0 mL of boiling water in a flask. The flask was closed and left under agitation for 5 minutes. The mixture was then allowed to cool to room temperature before analysis.

C 2.2.3 Dispersive Liquid-Liquid Microextraction

For the preliminary studies, to evaluate the acid used in the derivatization reagent solution preparation, the DLLME procedure started by adding the aqueous phase (5.0 mL) to the conical-bottom centrifuge tube. This aqueous phase included 4.5 mL of the standard solution of carbonyl compounds and IS, at 1 mg/L, and 500 µL of derivatization reagent PFPH (5.6 g/L in acid). Following, 1.3 mL of EtOH (dispersive solvent) and 90 µL of 1-octanol (extraction solvent) were mixed and rapidly added to the aqueous phase. The tube was then closed and placed in the ultrasonic bath (Fisherbrand, FB15055, Fisher Scientific) at 40 ºC for 30 minutes, for simultaneous extraction-derivatization. The tube was then centrifuged at 4000 rpm for 5 minutes (P Selecta, CENTROMIX-II model 700255, Spain), resulting in the formation of an extract drop at the top of the tube. The drop, containing the hydrazone-analytes, was collected using a 100 µL microsyringe (Hamilton, USA) to a separated vial before being analyzed by GC-MS.

For the following studies with coffee samples some modifications were performed in the DLLME procedure. A scheme of the optimized sample preparation and DLLME procedure is presented, in Figure 27. Briefly, a sample volume of 0.5 mL (prepared as described in section C 2.2.2) was added to 0.75 mL of EtOH (dispersive solvent) and vortex mixed (Hei-MIX multi Reax, Heidolph, Germany) for 1 minute. The mixture was centrifuged for 2 minutes at 3500 rpm. All supernatant was collected and mixed with 60

µL of isooctane (extraction solvent). The mixture was immediately and rapidly introduced into a glass conical centrifuge tube containing 150 µL of derivatization reagent PFPH (11.2 g/L in 10.4 mol/L H_3PO_4), and the IS, ACE-d4, at 1 mg/L. The tube was closed and placed in the ultrasonic bath at 50 ºC for 15 minutes. The subsequent steps were identical to those in the previous DLLME procedure.

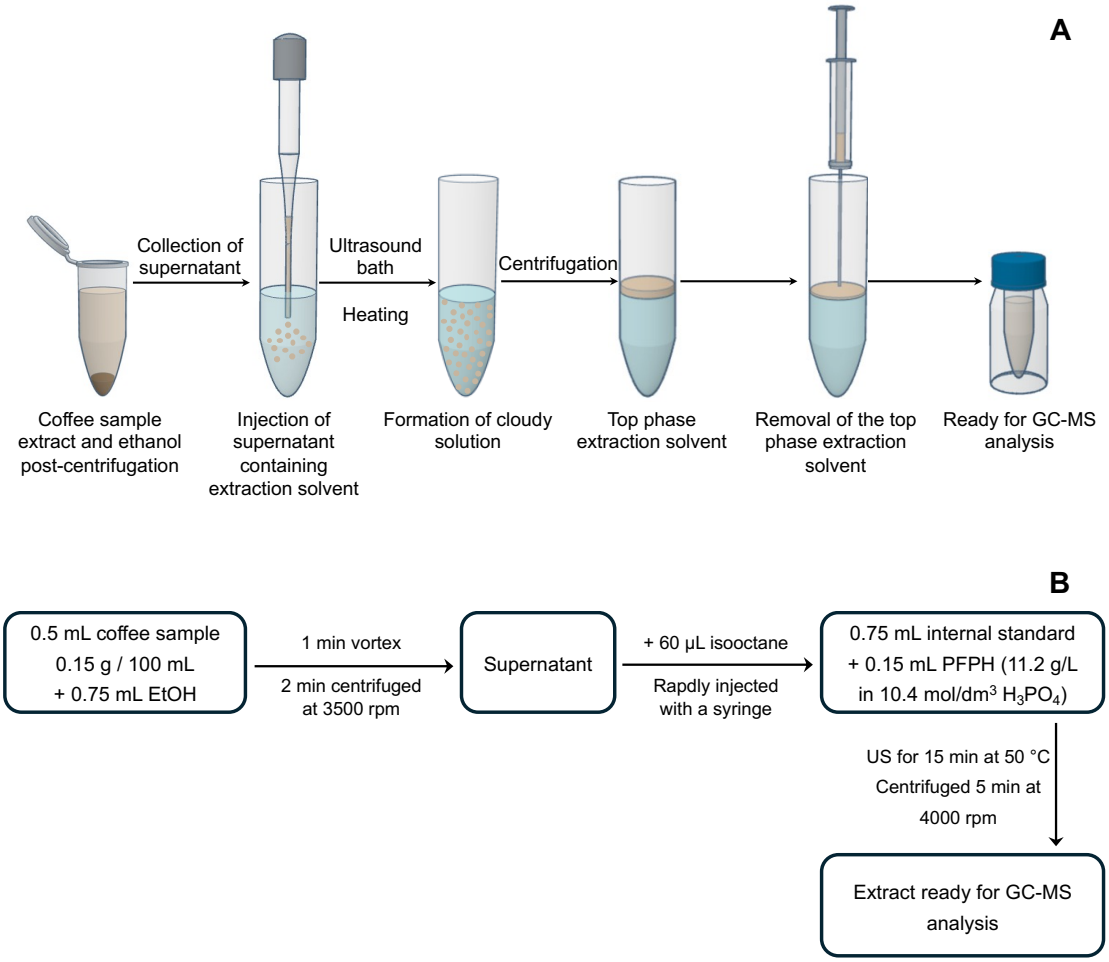


Figure 27 – A – Schematic representation of coffee sample preparation and DLLME extraction; B – Optimized conditions for coffee sample preparation and DLLME extraction.

C 2.2.4 GC-MS Analysis

Chromatographic separation and analyte identification were carried out using 7890B-5977B GC–MS system (Agilent Technologies, CA, USA). The injector, equipped with an ultra-inert double taper liner, was operated in splitless mode at a temperature of 270 ºC. A J&W HP-5MS column, Agilent Technologies, CA, US (30 m × 0.25 mm id × 0.25 µm) was used and the carrier gas used was helium, 1.0 mL/min.

The oven temperature program began at 80 ºC, increased at a rate of 10 ºC/min to 150 ºC, then increased at a rate of 30 ºC/min to 280 ºC, and held for 3 minutes, resulting in a total analysis time of 15.3 minutes. The transfer line, set at 280 ºC, connected the column to an electron ionization source operating in positive mode (EI +), at 230 ºC and 70 eV. A single quadrupole mass analyzer, set at 150 ºC, was used to perform a full scan (100-500 m/z) of each extracted hydrazone-analyte to identify the most intense ions. Based on this information, the selected ion monitoring (SIM) mode was used for hydrazone-analytes determination. A volume of 1 µL of the extract was injected for each analysis.

C 2.2.5 Method Validation

The analytical method was validated according to the FDA guidelines for the validation of chemical methods in food, cosmetics and veterinary products [261] and bioanalytical method validation guidance for industry [297], using regular coffee as the blank sample.

For each analyte, and the IS, a quantifier and two qualifier ions with different retention times were selected. The absence of interferents in the elution region of these analytes confirmed the method´s specificity.

To obtain the LOD and lower limit of quantification, a regular coffee sample, with low concentration of the targeted analytes, was used as blank and three levels of concentration were added. The limits were calculated by the average of the blank signal plus 3.3 or 10 times the standard deviation of the blank signal, for LOD or lower limit of quantification, respectively. The obtained signal values were converted in the corresponding concentrations.

The upper limit of quantification was set at 3 mg/L. Calibration curves with six concentration levels (n = 3), between the lower limit of quantification and upper limit of quantification, were performed to evaluate the method’s linearity.

C 2.2.6 Statistical Analysis

Experimental design plays an important role in optimizing research efficiency and effectiveness. By simultaneously varying multiple factors at different levels, it is possible to explore the main effects and interactions of these factors with fewer experiments. NemrodW ® statistical software (LPRAI, Marseille, France) was used to generate the experimental designs, evaluate the results, and plot the effects and response.

First, an asymmetrical factorial design ($2^3 3^1$ // 8) was used to screen four factors at different numbers of levels. Three variables (volume of the aqueous phase, dispersive solvent, and volume of the dispersive solvent) were studied at 2 levels, and one variable (derivatization reagent concentration) was studied at 3 levels, resulting in 8 runs. Secondly, a Doehlert experimental design was used to evaluate the influence of two other variables in detail (derivatization time and temperature in the ultrasonic bath). For two variables, the Doehlert design consists of six points forming a regular hexagon, plus 3 central points [298]. This design presents advantages compared to other experimental designs, such as, the ability to study variables with different numbers of levels, requiring fewer experiments, and enabling the exploration of the whole experimental domain [299]. Desirable functions were included in the experimental design methodology to identify optimal experiment conditions [300].

C 2.3 Results and Discussion

C 2.3.1 Optimization of Dispersive Liquid-Liquid Microextraction

In UA-DLLME, both the dispersive and extraction solvents are crucial for efficient analytes extraction from complex matrices and must meet specific criteria. The dispersive solvent, responsible for dispersing the extraction solvent in the aqueous sample, must be miscible with both aqueous and organic solvents [301]. EtOH, a green solvent previously used by Jain et al. [302] as a dispersive solvent for the extraction of parabens from different matrices (food, cosmetic and water), was chosen as a greener alternative to other conventional dispersive solvents, such as ACN. The extraction solvent must be immiscible or have low miscibility with water, have a high extraction capability for the target analytes, and be able to form an emulsion with the mixture dispersive solvent and aqueous phase [303]. Traditionally, these solvents were required to have a higher density than water. However, with the increasing demand for greener solutions, low-density and low toxicity solvents are becoming preferred for DLLME, such as n-hexane, 1-octanol, toluene, and isooctane [107,304]. Among this solvents, 1-octanol and isooctane were evaluated as extraction solvents in the present work. 1-Octanol has already proved to be a suitable alternative to chlorinated solvents [305] presenting low toxicity [306] and approved for human use by the FDA [307], easily biodegradable (according to the safety data sheet – Sigma Aldrich) and can be obtained through renewable resources [305]. Isooctane also presents low toxicity [308]. When compared to other commonly used solvents, Tobiszewski et al. [309] ranked isooctane

43rd out of 78 solvents (six out of sixteen non-polar solvents included) in their Environmental Risk Ranking of Solvents, indicating that while not ideal, its environmental impact is significantly lower than that of chlorinated solvents without exception.

C 2.3.1.1. Derivatization Reagent Solution Composition

A preliminary study assessed the impact of the acid in the derivatization reagent solution (PFPH) by comparing HCl and H_3PO_4. The PFPH stock solutions were prepared in 2.0 mol/dm^3 HCl and in 10.4 mol/dm^3 H_3PO_4 (as described in section 2.1), to maintain an equal pH value (0.7), during simultaneous extraction-derivatization. For this initial study, certain experimental conditions were selected based on prior experience. These included a total aqueous phase volume of 5.0 mL, 90 µL of 1-octanol, 1.3 mL of EtOH, 0.56 g/L of PFPH, and 40 ºC and 30 minutes for derivatization. The derivatization process was carried out in an ultrasonic bath under referred temperature and time settings. The extraction of carbonyl compounds from a model solution of carbonyl compounds, 1 mg/L, using PFPH solutions prepared using the aforementioned acids, was performed. The extracts were analyzed by GC-MS. A comparison was performed between the chromatographic peak areas obtained for each carbonyl compound when using each acid. Not all analytes presented the same behavior. GO and FUR presented lower response when using H_3PO_4, with a peak decrease of 20 to 50 %, when compared to the results when using HCl. For ACRL and ACE, better results were obtained when using H_3PO_4, with a small increase of 15 to 30 %, compared to the results when using HCl. For the remaining carbonyl compounds, an increase of peak area higher than 200 % is observed when using H_3PO_4, instead of HCl. Based on these results, the derivatization reagent solution was prepared with H_3PO_4.

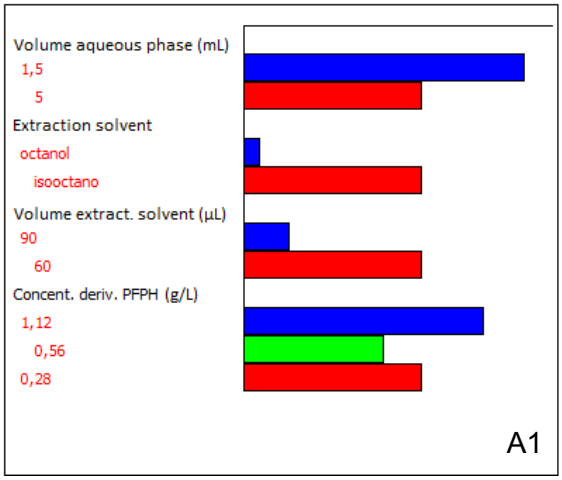
C 2.3.1.2. Experimental Designs

The optimization of UA-DLLME experimental conditions to enhance the simultaneous extraction-derivatization efficiency of carbonyl compounds from coffee samples was performed in two steps, using two different experimental designs. Optimization studies were performed using a sample of regular coffee, 0.60 g / 100 mL, prepared as described in section C 2.2.2, spiked with 1 mg/L of the 9 target analytes and 1 mg/L of IS.

An asymmetrical screening design $2^3 3^1$ // 8 was used to evaluate the influence of four parameters on the simultaneous extraction-derivatization effectiveness: b_1 – volume of the aqueous phase (1.5 mL and 5.0 mL); b_2 – type of extraction solvent (1-octanol and

isooctane); b_3 – volume of the extraction solvent (90 µL and 60 µL); and b_4 – PFPH concentration (1.12 g/L, 0.56 g/L and 0.28 g/L). The sample volume was maintained constant at 0.5 mL through all experiments. The volume of EtOH was adjusted in proportion to the aqueous phase volume to maintain a consistent aqueous-to-dispersant solvent ratio. Specifically, 0.75 mL of EtOH was used for an aqueous phase volume of 1.5 mL, while 1.3 mL of EtOH was used when the aqueous phase volume was increased to 5.0 mL.

The results for each carbonyl compound were plotted in Total Effect Plots and Delta Weight (Figure 28). In the Total Effect Plots, the bar length is proportional to the response (chromatographic peak area) obtained for each parameter, for example, according to the results in Figure 28-E1, the increase of aqueous phase volume in the extraction of DA halved the peak area. The Delta Weight plot represents the influence of changing a level of a parameter in the given response. For example, $b_{1/2\text{-}1}$ evaluates the impact of changing the volume of aqueous phase (b_1) from level 1 (1.5 mL) to level 2 (5.0 mL) in the chromatographic peak area. This response is also proportional to the bar length, and the side to which the bar goes shows a negative (left) or positive (right) effect. The dotted lines represent the statistical significance level (95 % confidence level), so an effect is considered statistically significant if the bar length exceeds the dotted lines. In Figure 28-E2, the increase of the aqueous volume phase ($b_{1/2\text{-}1}$), from 1.5 mL to 5.0 mL, presents a negative effect in the simultaneous extraction of DA. In contrast, a positive effect is presented with the reduction of extraction solvent volume ($b_{3/2\text{-}1}$), from 90 to 60 µL. This reduction of extraction solvent enhances the enrichment factor, as more efficient analyte extraction was achieved within the smaller volume of the extraction solvent droplet collected at the end of the procedure.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0,28
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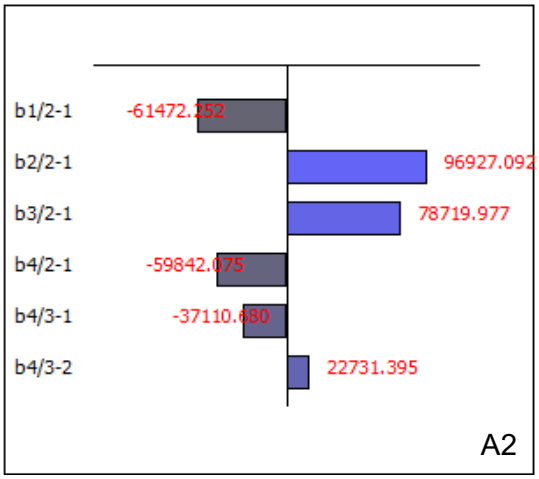
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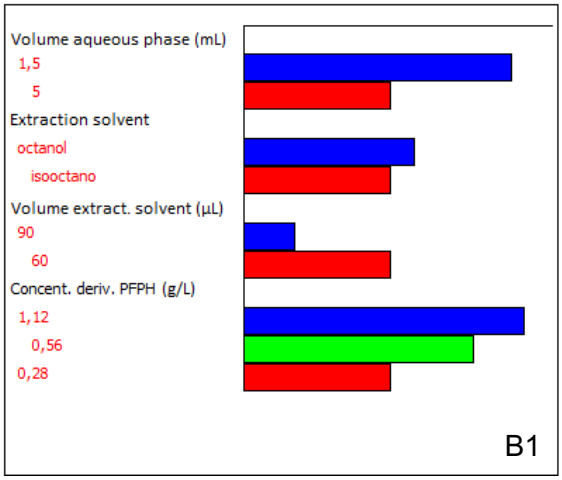
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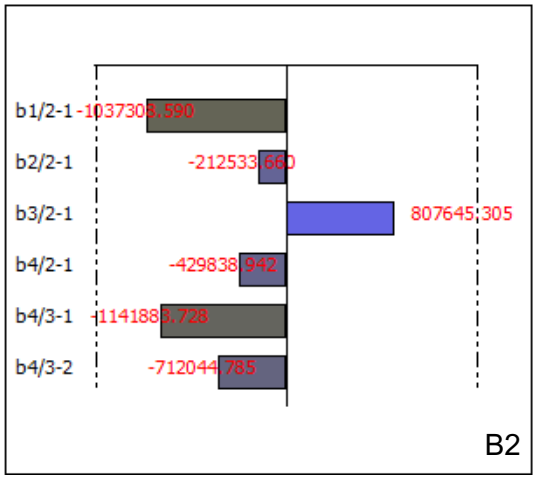
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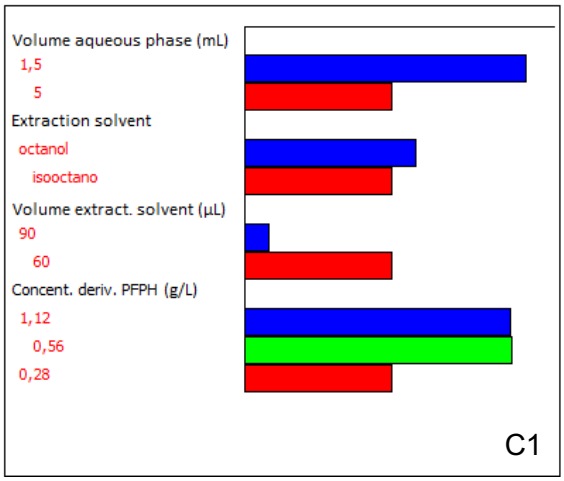
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Extraction solvent
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Volume extract. solvent (µL)
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Concent. deriv. PFPH (g/L)
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0,28
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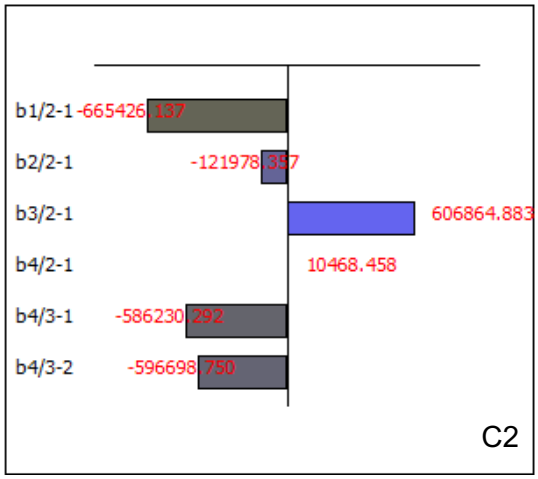
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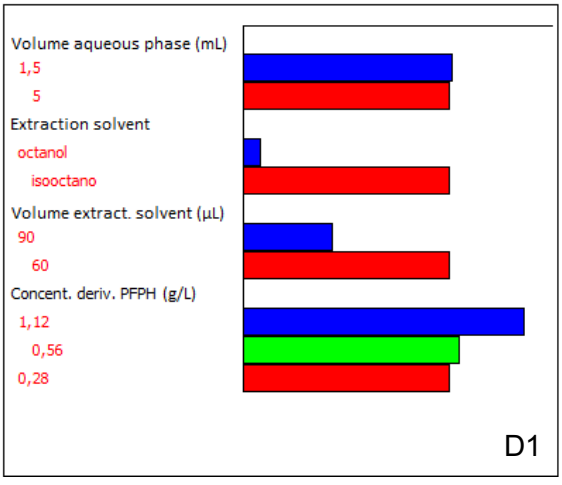
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Volume extract. solvent (µL)
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Concent. deriv. PFPH (g/L)
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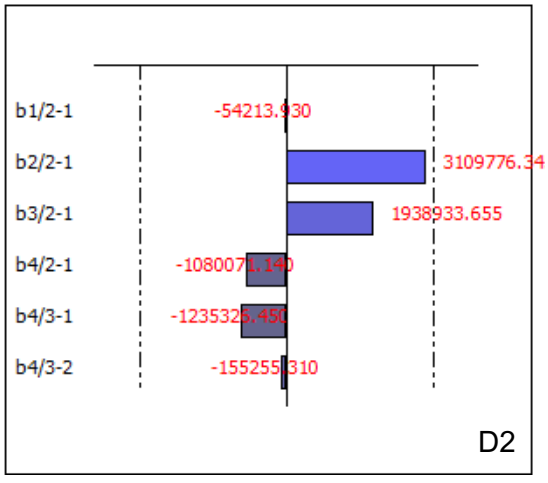
0,56
0,28
D1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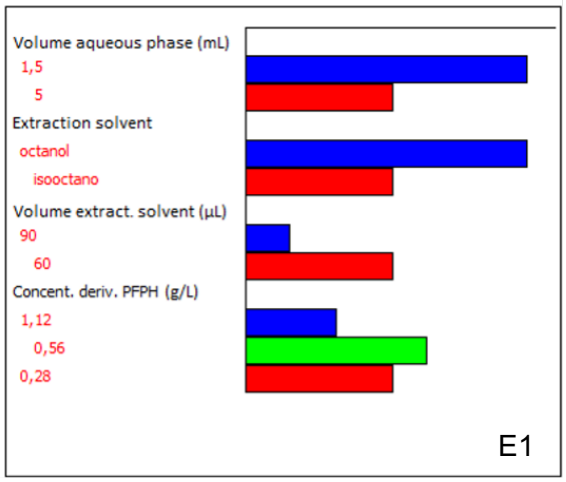
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Volume extract. solvent (µL)
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Concent. deriv. PFPH (g/L)
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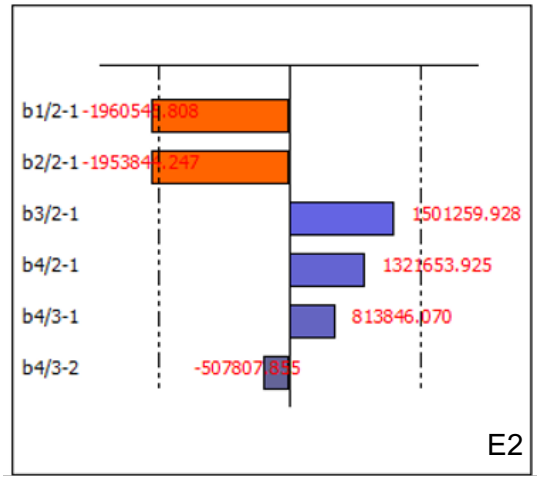
0,56
0,28
E1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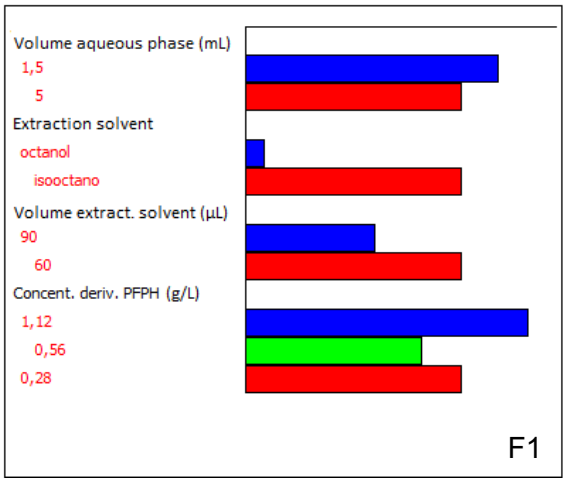
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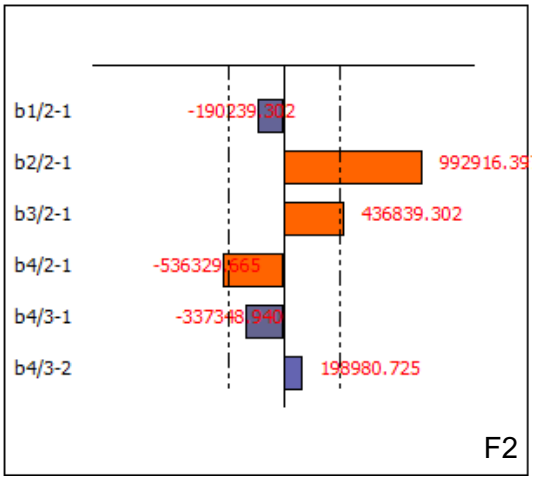
0,28
F1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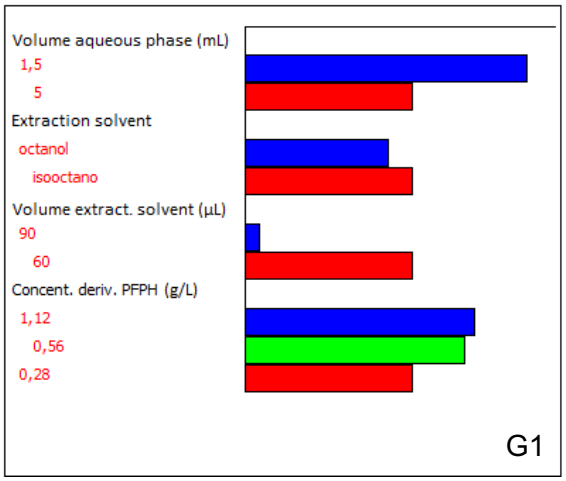
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0,28
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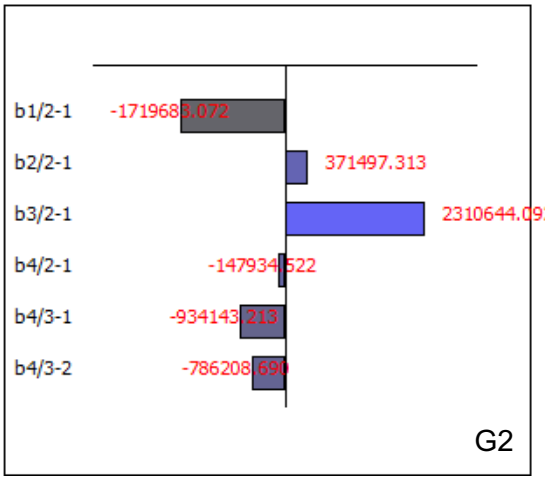
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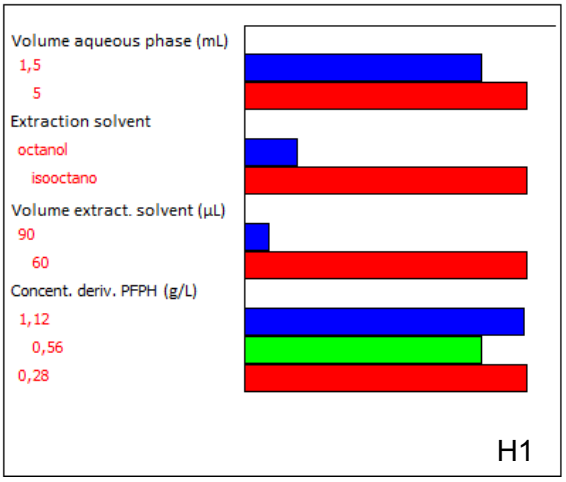
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Extraction solvent
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Volume extract. solvent (µL)
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Concent. deriv. PFPH (g/L)
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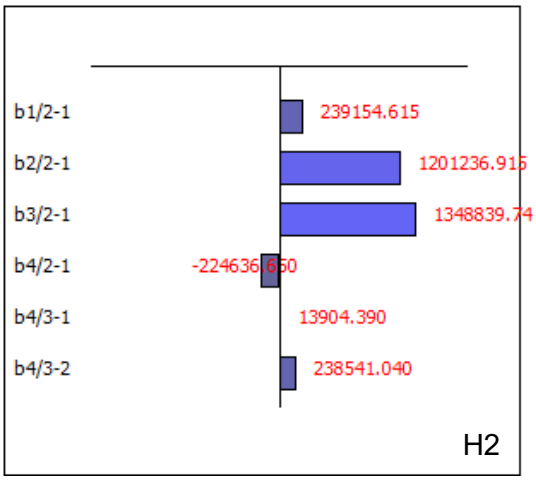
0,56
0,28
H1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H2

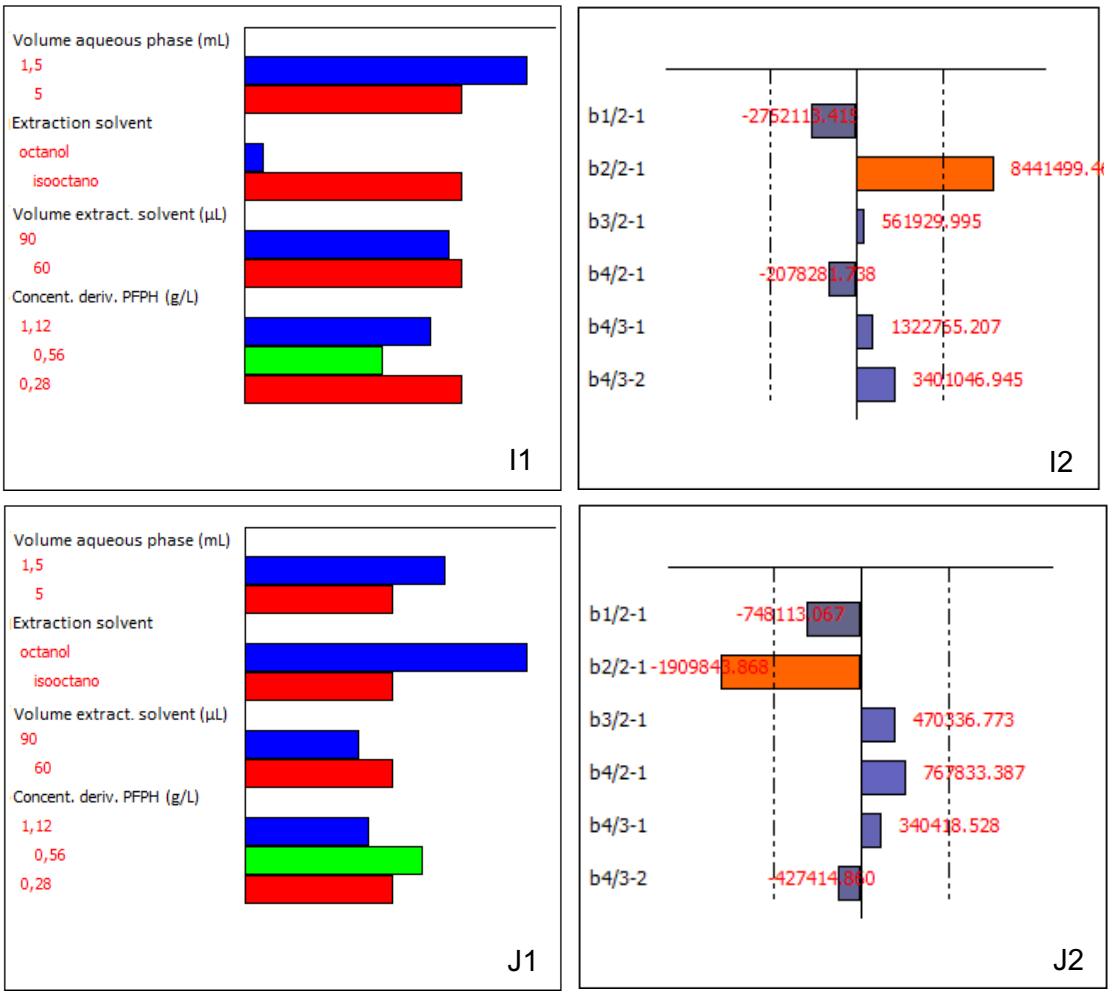


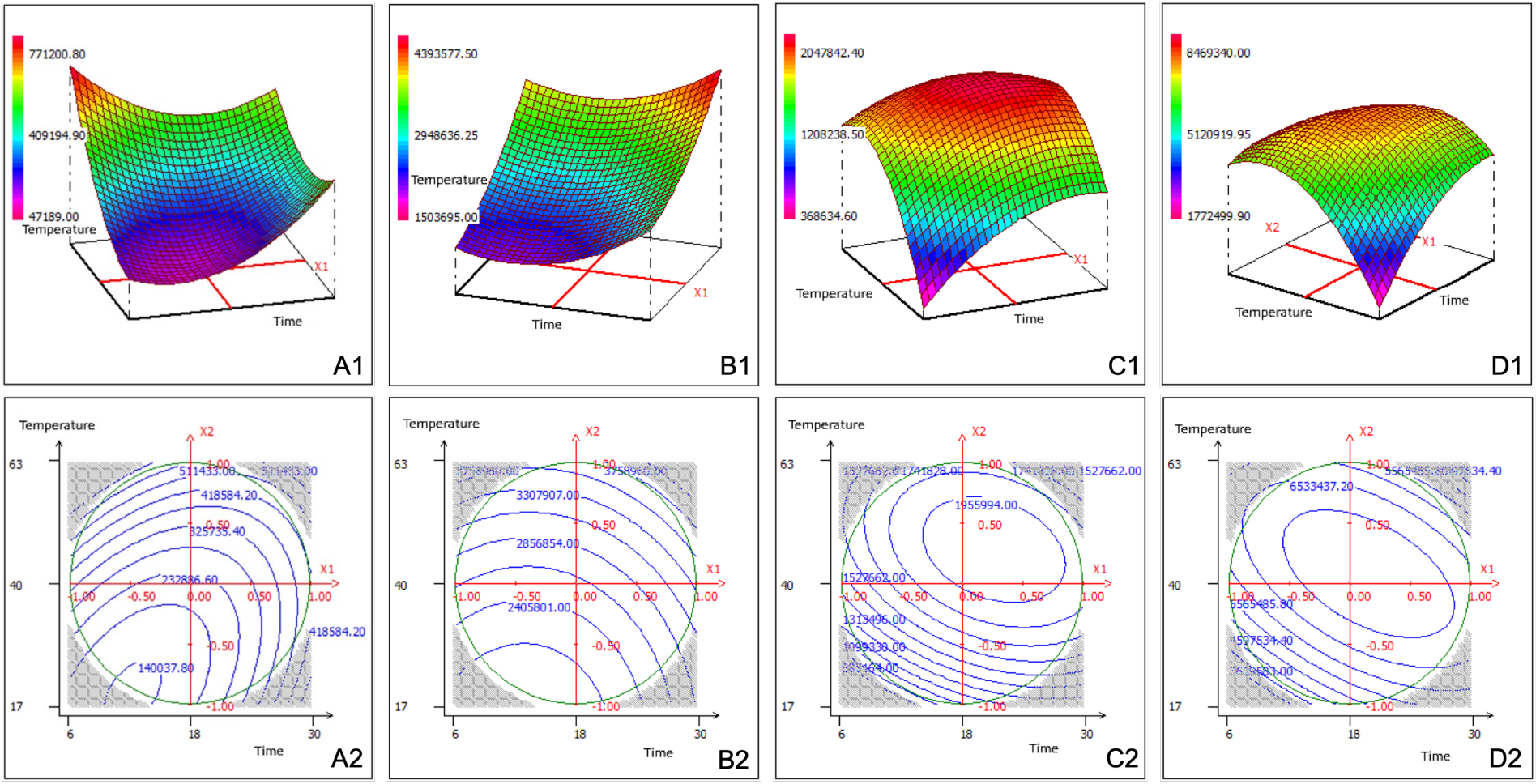
Figure 28 – Total effects (1) and delta weights (2) plots of the asymmetrical screening design $2^{3}3^{1}$ // 8 for A – ACE-d4; B – ACE; C – ACRL; D – PhCHO; E – DA; F – FCHO; G – FUR; H – GO; I – MDA; and J – MGO.

Based on the obtained Delta Weight and Total Effect plots, the extraction solvent volume that presents better results is 60 µL, for all carbonyl compounds. A lower aqueous phase volume, 1.5 mL, shows higher values for almost all the studied analytes except for GO. Regarding the type of extraction solvent used, isooctane is a better choice for 5 (ACE-d4, PhCHO, FCHO, GO and MDA) of the 10 carbonyl compounds, for 3 (ACE, ACRL and FUR) of the remaining 5 both extraction solvents present similar results, and only for the other 2 (MGO and DA) does 1-octanol present better results. Also, PFPH concentration does not present a consensual response for all analytes. However, 1.12 g/L is the concentration with better results overall.

Ultimately, based on the presented plots and obtained results, the following optimized parameters were selected: 60 µL of isooctane (type and volume of extraction solvent), 1,5 mL (volume of aqueous phase), and 1.12 g/L (PFPH concentration).

Following the optimization steps, a two-variable Doehlert experimental design was applied to optimize carbonyl compounds' derivatization time and temperature with PFPH. The derivatization time was studied at 5 levels (6, 12, 18, 24, and 30 minutes), and the derivatization temperature was studied at 3 levels (20, 40, and 60 ºC). The software generates 9 runs, 3 of them were performed in the same conditions, representing the central point of the design. The experiments were also performed in a random order according to the software instructions.

A surface response plot was obtained for each carbonyl compound (Figure 29). Most of the analytes showed similar behaviors, presenting better results with conditions near the central point (18 minutes at 40 ºC). Only MGO, ACE, and ACE-d4 showed different results. The surface response plot in Figure 30 shows the graph of the multi-criteria decision analysis, based on desirability functions, used to predict the potential optimal conditions. The best results created a plateau in the surface response plot, showing a correlation between time and temperature; a decrease in derivatization time is needed to obtain similar results with an increase in derivatization temperature. For example, the results obtained for 15 minutes of derivatization at 50 ºC should be similar to the results obtained for 25 minutes of derivatization time at 40 ºC. Based on these results, a lower time and higher temperature (15 minutes, 50 ºC) were chosen as the optimized parameters for the following studies.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Time
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X1
Time
B1
2047842.40
1208238.50
368634.60
X1
Temperature
Time
C1
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5120919.95
1772499.90
X2
X1
Temperature
Time
D1
Temperature
X2
63
40
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Time
A2
Temperature
X2
63
40
17
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6
18
30
Time
B2
Temperature
X2
63
40
17
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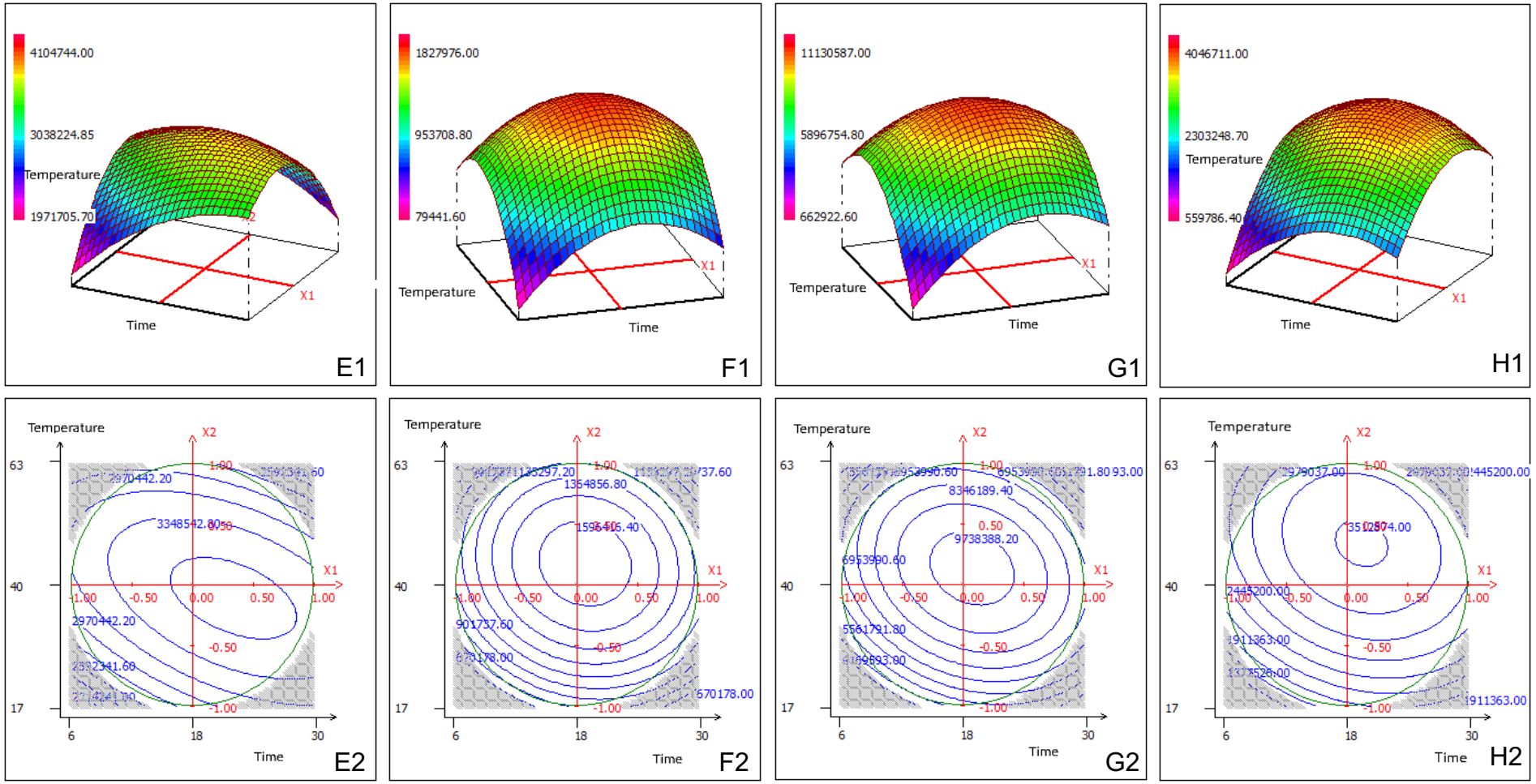
30
Time
D2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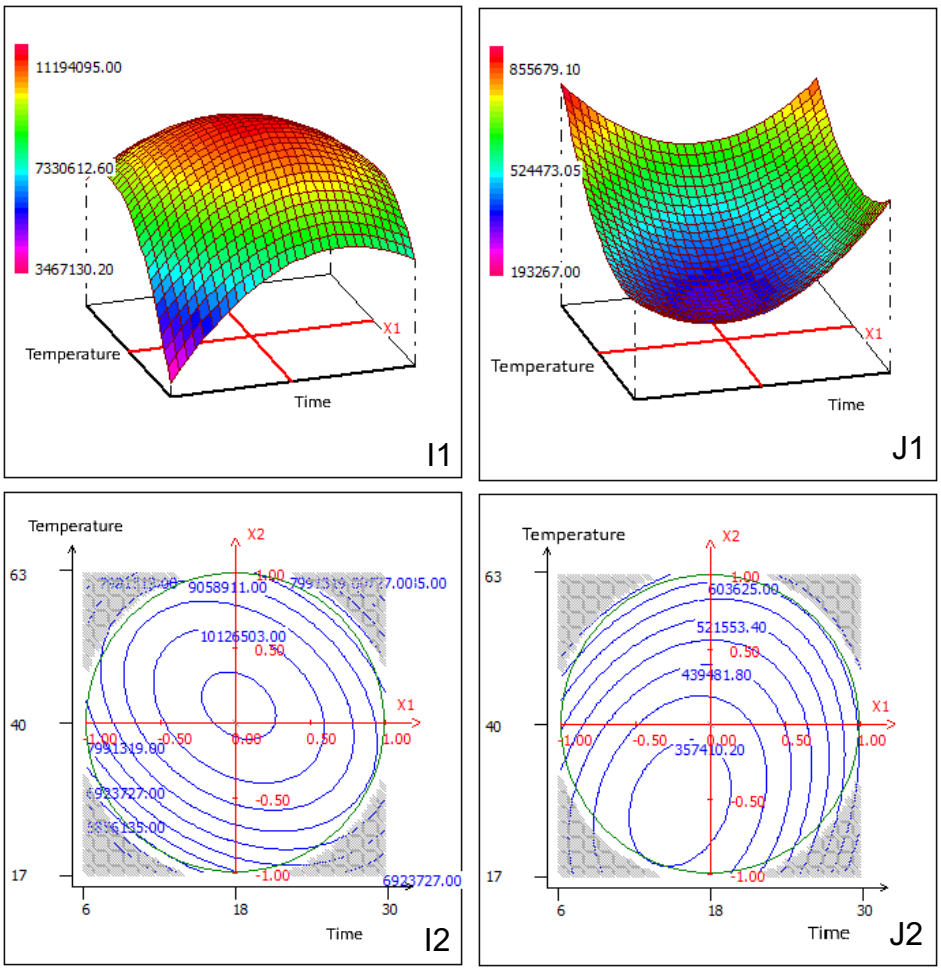

Figure 29 – Surface response plot tridimensional (1) and bidimensional (2), for a two-variable Doehlert design regarding time and temperature of derivatization, for A – ACE-d4; B – ACE; C – ACRL; D – PhCHO; E – DA; F – FCHO; G – FUR; H – GO; I – MDA; and J – MGO.

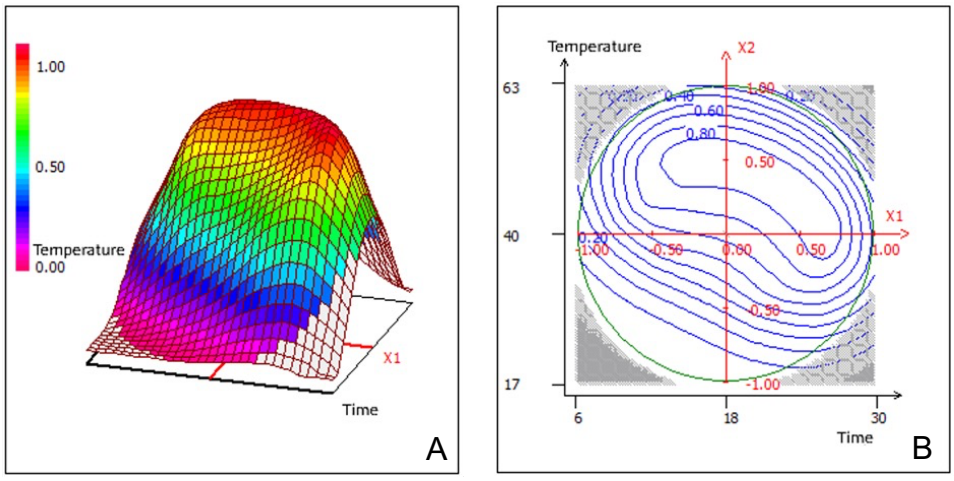

Figure 30 – Surface response plot: A – three-dimensional, B – Two-dimensional; for a two-variable Doehlert design regarding time and temperature of derivatization for all the target carbonyl compounds.

C 2.3.2. Analytical Validation

The developed methodology was validated according to the FDA guidelines, and the results of the evaluated parameters are summarized in Table 25 and Table 26. Method specificity and selectivity were evaluated by selecting a quantifier and two qualifier ions for each analyte, each with different retention times. As observed in Figure 31 and Table 25, the carbonyl compounds have different quantifier ions and retention times. No interferences were present in the GC-MS chromatograms of the coffee samples.

Table 25 – Parameters to evaluate the method's specificity, sensitivity and linearity, and the limits of detection and quantification, for the determination of carbonyl compounds in coffee samples.

Analyte	RT	Quantifier ion	Qualifier ion		r^2	LOD	LLOQ#
	min	m/z	m/z	m/z		µg/L	µg/L
FCHO	5.266	210	211	162	0.9997	377.9	388.7
MDA	5.462	234	117	180	0.9995	348.3	348.4
ACE-d4*	6.346	228	227	229	-------	-------	-------
ACE	6.395	224	225	223	0.9993	383.0	421.9
ACRL	7.671	236	115	235	0.9991	304.1	304.5
DA	9.586	266	267	155	0.9991	299.7	300.9
MGO	9.783	252	155	182	0.9993	271.1	289.0
FUR	10.841	276	277	181	0.9998	362.2	370.4
PhCHO	11.452	286	405	327	0.9990	374.6	375.6
GO	12.483	235	131	183	0.9991	435.8	436.1

*Used as IS / #LLOQ – Lower Limit of Quantification

Table 26 – Accuracy, precision and matrix effect studies of UA-DLLME-GC-MS methodology for the determination of 9 carbonyl compounds in coffee samples.

Analyte	Accuracy			Precision						Matrix effect	
	% Recovery (n =3)			Intraday (n = 5)			Interday (n = 5)				
	500 µg/L	1000 µg/L	2000 µg/L	500 µg/L	1000 µg/L	2000 µg/L	500 µg/L	1000 µg/L	2000 µg/L	F-test	*t*-test
FCHO	5.266	210	211	5.71	3.54	3.25	4.24	4.74	3.10	1.75	2.57
MDA	5.462	234	117	3.57	6.28	4.27	4.84	7.82	4.20	2.14	2.19
ACE-d4*	6.346	228	227	8.37	4.39	4.91	9.56	5.37	6.91	3.27	1.25
ACE	6.395	224	225	6.08	5.85	5.64	6.80	9.03	8.23	3.80	0.14
ACRL	7.671	236	115	5.49	3.42	2.67	7.78	4.30	2.59	2.99	0.54
DA	9.586	266	267	5.52	7.83	5.17	5.80	8.78	9.03	1.62	0.76
MGO	9.783	252	155	2.85	6.24	4.75	7.35	7.77	5.40	4.56	1.62
FUR	10.841	276	277	8.05	3.90	5.63	8.49	8.79	9.43	1.23	1.04
PhCHO	11.452	286	405	6.46	6.98	4.24	8.78	8.35	3.26	2.49	0.51
GO	12.483	235	131	5.71	3.54	3.25	4.24	4.74	3.10	1.75	2.57

*Used as IS
Intraday and interday studies are presented as relative standard deviation (%)
t_{critic} = 3.18 (95 %, υ = 4); F_{critic} = 18.51 (95 %, υ_1 = 1 and υ_2 = 2)

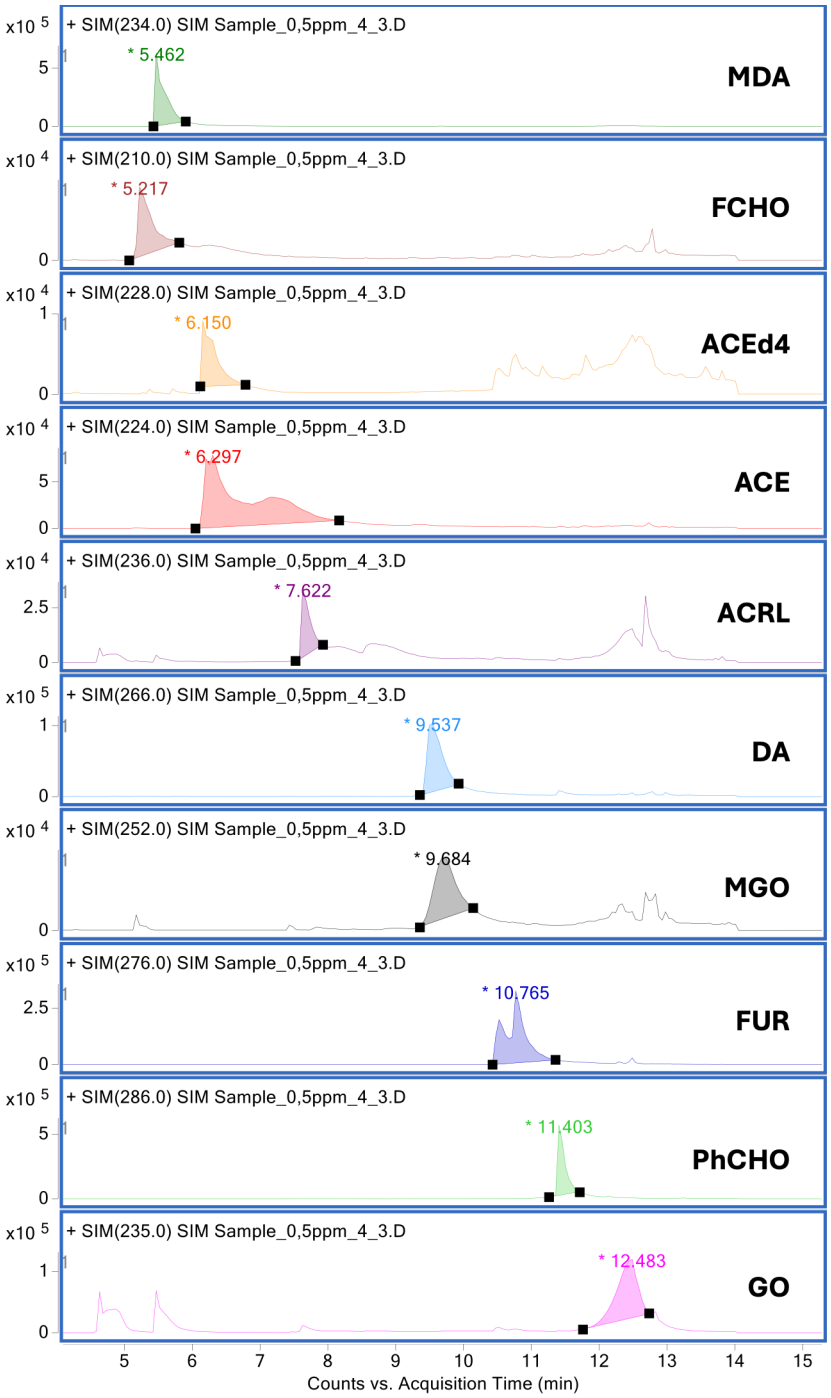

Figure 31 – Chromatogram of 0.5 mg/L of MDA, FCHO, ACE, ACRL, DA, MGO, FUR, PhCHO and GO extracted by UA-DLLME from coffee. ACE-d4 was used as IS at of 1 mg/L.

Linearity, limits of detection and quantification, and sensitivity for the target analytes are summarized in Table 25. ACE-d4 was used as IS, with a concentration of 1000 µg/L. Using the standard addition method, with IS, a concentration range of 300 µg/L to 3000 µg/L was studied for all target analytes, except for ACE, for which the range started at 500 µg/L. The calibration curves obtained showed excellent linearity, with $r^2 \geq$

0.9990. In Table 25, the LOD and lower limit of quantification are discriminated for all target analytes, while the upper limit of quantification was set at 3000 µg/L.

Matrix effect, accuracy and precision are summarized in Table 26. The matrix effect was evaluated by performing the statistical comparison of the slopes obtained for the calibration curve method and the standard addition method [228], for each analyte, using three concentration levels (n = 3). The values obtained for the F-test and *t*-test, compared to the critical values, indicated that no significant matrix effect is observed in the developed methodology for the extracted carbonyl compounds from coffee samples. The standard addition method was the chosen one since coffee samples with different and more complex compositions were also studied. The standard addition method was used to evaluate the recovery (n = 3) and intraday and interday precision, measured in terms of % RSD (n = 5). Both were performed in 3 levels of concentration. Recovery values between 90 % and 110 % were obtained. Intraday precision was less than 8.4 % while interday precision was less than 9.6 %.

C 2.3.3. Greenness Assessment

The developed methodology presents a greener alternative for extracting carbonyl compounds from coffee samples, using the US-DLLME technique.

AGREEprep open access software [310] was used to evaluate the environmental and safety impact of the developed methodology. This tool scores the methodologies according to the principles of green chemistry, evaluating parameters such as reagents' toxicity, waste generation, energy consumption and sustainability. The methodology is assessed across 10 criteria, each receiving a score from 0 (poorest performance) to 1 (optimal performance). A final score (0 to 1) is calculated considering the default weights for each criterion. The developed UA-DLLME-GC-MS methodology achieved an AGREEprep score of 0.59 (Figure 32). The individual criterion scores are described in Table 27. The overall positive score reflects the methodology's favorable environmental and safety performance, with six of the ten criteria scoring above 0.5. Notably, criterion 8 received a high score due to the low energy consumption *per* analysis, attributed to the use of an ultrasonic bath, which enables the simultaneous preparation of multiple samples. Highlighting the method's alignment with green chemistry principles. Only two criteria presented scores below 0.5, post-sample preparation configuration for analysis (criterion 9) and operator's safety (criterion 10).

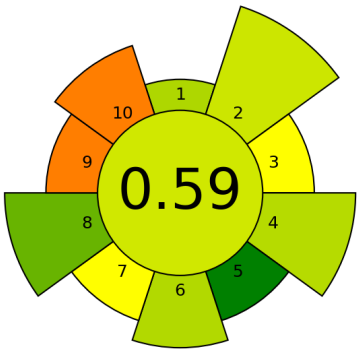

Figure 32 – Graph of AGREEprep assessment of the environmental impact associated with the methodology's sample preparation.

Table 27 – AGREEprep report for the methodology's sample preparation. Number (#), description (as presented in the metric tool), score and weight of each criterion.

#	Criterion	Score	Weight
1	**Sample preparation placement** On-line/In-situ	0.66	1
2	**Hazardous materials** (Mass [g] or volume [mL] of problematic materials) 0.1565	0.6	5
3	**Sustainability, renewability, and reusability of materials** 50-75% of reagents and materials are sustainable or renewable, but can only be used ONCE	0.5	2
4	**Waste** (Mass [g] or volume [mL] of waste) 0.918	0.64	4
5	**Size economy of the sample** (Mass [g] or volume [mL] of the sample) 0.003	1.0	2
6	**Sample throughput** (Hourly sample throughput) 16	0.65	3
7	**Integration and automation** (No. of sample preparation steps / degree of automation) 2 steps of fewer / Semi-automated systems	0.5	2
8	**Energy consumption** (Approximate energy consumption *per* analysis [W]) 22	0.8	4
9	**Post-sample preparation configuration for analysis** Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10	**Operator's safety** (No. of distinct hazards) 3 hazards	0.25	3

In order to contextualize the current methodology, Table 28 summarized key features from other methodologies, reported in the literature, employing the DLLME technique for the extraction of carbonyl compounds from food matrices, offering a basis for comparison.

Firstly, it is important to highlight that many reported methodologies require additional sample preparation steps before or after the DLLME process. These steps include pH adjustment, derivatization, or dilution in an organic solvent. However, two of the reviewed methodologies incorporate the derivatization reaction in the extraction procedure [260,311], as does the present work, thereby decreasing the overall sample preparation time.

Some of the presented methodologies use high-density solvents, such as $CHCl_3$ and CH_2Cl_2 as extraction solvents and ACN as the dispersive solvent, all of them not included in the green solvents category [312,313]. Nevertheless, other studies present promising green alternatives [311,314,315], introducing deep eutectic solvents (DES), ionic liquids, and 1-octanol as extraction solvents. Regarding the dispersive solvent, only two methodologies use green solvents [315,316], namely butan-1-ol and EtOH. Of the presented methods, only one [315], employs both extraction and dispersive solvents, which are categorized as green solvents.

Regarding the analysis following the DLLME process, most of the methodologies use liquid chromatography coupled with spectrophotometric detection [311,314,315]. An exception is the determination of GO, which employs fluorometric detection [316]. Meanwhile, two works use GC-MS [260,289] as does the present study. GC-MS is considered a greener chromatographic technique in terms of solvent consumption and waste generation compared to liquid chromatography.

Table 28 – Published methodologies for the extraction of carbonyl compounds targeted in this work, from food matrices, using DLLME technique.

Carbonyl compound	Food matrix	Sample preparation pre-DLLME	Steps included in DLLME		Extraction solvent		Dispersive solvent		Instrument for analysis	Ref
			Steps	Time	Solvent	Volume (µL)	Solvent	Volume (µL)		
MDA, DA, FCHO, MGO, ACE, FUR, ACRL, PhCHO, GO	Coffee	Interferent precipitation with dispersive solvent	Ultrasound-assisted derivatization and extraction	15 min	Isooctane	60	EtOH	750	GC-MS	Current work
			Centrifugation	5 min						
FCHO, ACE, (5 more aldehydes)	Drinking water, Alcoholic beverages	Filtration, pH adjustment, Derivatization[a]	Centrifugation	1 min	DES	100	ACN	700	HPLC-UV/Vis	[314]
ACE, ACRL, FCHO, MDA, (2 more aldehydes)	Edible oils	GDME with derivatization[a] and ultrasound agitation	Sonification	5 min	$CHCl_3$	70	ACN	750	GC-MS	[289]
			Centrifugation	2 min						
FCHO	Beverages	Dilution, pH adjustment, Filtration	Microwave-assisted derivatization[a] and extraction	90 s	Ionic liquid	70	ACN	400	HPLC-UV/Vis	[311]
			Centrifugation[#]	10 min						
MDA, ACRL, 4-Hydroxy-2-nonenal	Beverages	Interferent precipitation with dispersive solvent	Ultrasound-assisted derivatization[a] and extraction	5 min	$CHCl_3$	90	ACN	1300	GC-MS	[260]
			Centrifugation	2 min						
GO[$]	Alcoholic beverages	pH adjustment, Interferent precipitation with dispersive and extraction solvents	Vortex	1 min	CH_2Cl_2	150	Butan-1-ol	750	HPLC-FD	[316]
			Centrifugation[$]	5 min						
FUR, Hydroxymethylfurfural	Baby formula	Hydrolyzation, Saponification, pH adjustment, Protein precipitation	Shaking	2 min	1-octanol	60	EtOH	650	HPLC-UV/Vis	[315]
			Centrifugation	2 min						

[a] DNPH as derivatization reagent

After extraction the ionic liquid phase was dissolved in 100 µL ACN

$ After extraction the upper phase was collected, the pH adjusted, and MeOH was added before analysis.

Compared to the presented methodologies, the developed methodology offers notable advantages for determining carbonyl compounds in food matrices. It allows the extraction and determination of a wider range of carbonyl compounds, including PhCHO, for which no DLLME-based methods for food matrices have been reported. Furthermore, this work highlights the feasibility of extracting carbonyl compounds using green solvents in the DLLME procedure.

C.2.3.4. Carbonyl Compounds Occurrence in Coffee Samples

The developed methodology was applied to the determination of the target carbonyl compounds in 13 coffee samples. The samples were analyzed in triplicate and the standard addition method with IS was used for analyte quantification. The results obtained are presented in Table 29.

Table 29 – Quantification of the studied analytes in different coffee samples. All results are presented in µg/L.

Coffee sample	FCHO	MDA	FCHO	ACE	ACRL	DA	MGO	FUR	PhCHO	GO
RC1	NQ	NQ	NQ	602 ± 33	NQ	ND	ND	1623 ± 62	ND	NQ
RC2	NQ	NQ	NQ	613 ± 31	NQ	ND	ND	NQ	ND	NQ
RC3	NQ	NQ	NQ	591 ± 38	NQ	ND	ND	NQ	ND	NQ
RC4	NQ	NQ	NQ	538 ± 33	NQ	ND	ND	NQ	834 ± 1	NQ
RC5	NQ	NQ	NQ	541 ± 34	NQ	ND	ND	NQ	764 ± 31	NQ
RC6	NQ	NQ	NQ	631 ± 31	NQ	ND	ND	2659 ± 122	583 ± 50	NQ
MC1	NQ	NQ	NQ	601 ± 35	NQ	ND	ND	2650 ± 147	ND	NQ
MC2	NQ	NQ	NQ	597 ± 36	NQ	ND	ND	1890 ±146	ND	NQ
MC3	NQ	NQ	NQ	574 ± 32	NQ	ND	ND	2250 ± 125	418 ± 24	NQ
MC4	NQ	NQ	NQ	622 ± 31	NQ	ND	ND	NQ	585 ± 37	NQ
MC5	NQ	NQ	NQ	597 ± 36	NQ	ND	ND	NQ	930 ± 57	NQ
RDC1	NQ	NQ	NQ	611 ± 31	NQ	ND	ND	NQ	NQ	NQ

NQ – detected, but not quantifiable

ND – not detected

ACE was detected at similar concentrations (541 to 631 µg/L) in all samples. PhCHO was quantifiable in 3 regular coffee samples (583 to 834 µg/L) and 3 of the mixture coffee samples (418 to 930 µg/L), but its concentration in decaffeinated coffee

samples was below the lower limit of quantification. FUR quantification was possible in 2 of the regular coffee samples (1623 to 2659 µg/L) and 3 of the mixture coffee samples (1890 to 2650 µg/L). GO, FCHO, MDA and ACRL were detected in all samples, however concentrations were below the lower limit of quantification. MGO and DA were not detected in any of the samples.

Table 30 – Analytical methods for the determination of carbonyl compounds in coffee samples.

Sample	Analytes	Concentration range (µg/g)	Sample preparation	Instrument	Ref
Instant coffee	MDA	0.17-2.12	TBA test	HPLC-UV	[317]
Natural and roasted coffee	MDA ACRL	0.6-1.5 0.1-0.81	US-DLLME with DNPH derivatization	GC-MS	[260]
Coffee green beans (different roasting degrees)	FCHO ACE GO MGO DA FUR PhCHO	0.5-0.9 5.4-10.6 1.2-1.7 0.4-18.7 10.3-23.7 9.5-576.7 9.3-22.3	LLE and DNPH derivatization	UHPLC-Orbitrap-MS	[318]
Robusta coffee beans	PhCHO	21.6	HS-SPME[$]	GC-MS	[319]
Coffee beverage	FCHO ACE	0.24-1.5 0.8-20.1	HS-SPME and PFBHA[+] derivatization	GC-MS	[320]
Chinese coffee products	FUR	2.6-49.2	UAE and dSPE[#]	GC-MS	[321]
Roasted coffee	FUR	109-200	Acidic aqueous extraction	UHPLC-UV	[322]
Roasted coffee	GO MGO DA	0.23-2.66 0.07-2.4 0.03-0.36	Maceration	HPLC-DAD	[76]
Arabica coffee	GO MGO DA	0.81-7.74 18.53-89.08 0.43-6.74	Water extraction	GC-NPD	[238]
Coffee extracts	MDA FCHO ACE ACRL DA MGO FUR PhCHO	<LOQ <LOQ 0.54-0.61 <LOQ <LOQ <LOQ 1.6-2.6 0.4-0.9	US-DLLME and PFPH derivatization	GC-MS	This work

+ PFBHA[+], O-(2,3,4,5,6-pentafluoro-benzyl)-hydroxylamine

dSPE, Dispersive solid-phase extraction

Table 30 compares carbonyl and dicarbonyl compounds reported in previous studies. The results indicate that higher levels of GO, MGO, and DA were found in earlier studies compared to this work. Chen et al. [318] reported GO (1.2-1.7 µg/g), MGO (0.4-18.7 µg/g), and DA (10.3-23.7 µg/g) in coffee green beans with different roasting degrees using LLE and DNPH derivatization, followed by UHPLC-Orbitrap-MS analysis. Similarly, Papetti et al. [76] found GO (0.23-2.66 µg/g), MGO (0.07-2.4 µg/g), and DA (0.03-0.36 µg/g) in roasted coffee, while Lee et al. [238] reported GO (0.81-7.74 µg/g), MGO (18.53-

89.08 µg/g), and DA (0.43-6.74 µg/g) in Arabica coffee beans soaked in acidic media before preparation. Furfural concentrations were also higher in roasted coffee (109-200 µg/g, [322]) than in this study's values. However, similar levels of FCHO and ACE were found in coffee beverages by Jeong et al. [320], and similar FUR concentrations were reported by Liu et al. [321] in Chinese coffee products, aligning with the results obtained here. The higher levels of carbonyl and dicarbonyl compounds observed in previous studies are likely due to processing conditions, such as coffee bean roasting and acidic treatment before preparation, which promote the formation and release of these compounds. The consistency of the results obtained in this study with those from previous research supports the robustness of the developed methodology for the simultaneous determination of carbonyl compounds in coffee extracts.

C.2.4. Conclusion

The developed UA-DLLME-GC-MS methodology allowed for the simultaneous extraction-derivatization of 9 carbonyl compounds (MDA, FCHO, ACE, ACRL, DA, MGO, furfural, benzaldehyde and GO) from coffee extracts. The sample preparation step was optimized through studies of key parameters using two experimental designs.

The developed methodology presented a greener alternative to previous reported methodologies, with isooctane and EtOH as extraction and dispersive solvent, respectively, and an AGREEprep score of 0.59.

Validation according to FDA guidelines demonstrated excellent selectivity, specificity, sensitivity, linearity and accuracy, with no matrix effect and recovery values ranging between 90 and 110 % for all analytes.

Three of the studied analytes were quantified in all coffee samples, acetaldehyde, benzaldehyde and furfural. Benzaldehyde was only quantifiable in 6 of the analyzed coffee samples, while furfural was only quantifiable in 5.

PART D

General Conclusions and Future Perspectives

In the field of Analytical Chemistry, there is a continuous demand for approaches that provide high sample analysis throughput, lower reagent consumption, and higher selectivity and sensitivity. In this context, the research presented in this thesis contributes to the advancement of sustainable analytical strategies by exploring simplified chromatographic systems and greener sample preparation methodologies for the determination of targeted analytes in complex food matrices.

The first core contribution of this thesis lay in the development and application of tailored low-pressure chromatographic flow systems with electrochemical detection for the determination of analytes in coffee samples. Several achievements were observed. Through the use of multiple pulse amperometry technique, it was possible for the first time, the parallel determination of oxidizable and reducible compounds (namely caffeine, trigonelline, and chlorogenic acids) during a sole chromatographic run without compromising resolution. Furthermore, the applicability of low-pressure chromatographic flow systems was expanded through the use of short-length (0.5 cm) phenyl monolithic columns for the separation of α-dicarbonyl compounds. In this case-study, the sample treatment performed before the chromatographic analysis was based on a novel extraction strategy for volatile compounds from liquid samples – the fan-assisted extraction and the full evaporation technique – which allowed the quantitative extraction of the volatile analytes while minimizing matrix interference and solvent consumption. These developments demonstrated that reduced instrumentation requirements do not compromise the analytical capability. Furthermore, these developments reinforce the versatility and capability of low-pressure chromatographic flow systems equipped with electrochemical detectors for analysing complex matrices while maintaining low operational costs, reduced solvent consumption, and low instrumentation requirements, a particularly attractive feature in routine monitoring contexts or resource-constrained laboratories.

The second core contribution of this thesis focused on the development of microextraction-based sample preparation approaches for the determination of volatile organic compounds in food matrices, prior to GC-MS analysis. The presented works illustrate a transition from traditional DLLME strategies to a greener approach, demonstrating that environmental impact can be significantly reduced without compromising analytical performance. Initially, lipid peroxidation products and VOCs in adult nutritional formulas were characterized using UA-DLLME and HS-SPME, showing the influence of formulation and storage conditions on the contaminant profiles and underscoring the importance of such monitoring for consumer safety. Subsequently, a greener UA-DLLME methodology was developed by replacing halogenated solvents with

lower-toxicity alternative ones. The environmental performance of the developed methodology was validated by the AGREEprep metric. This work highlights that greener approaches still require careful optimization as solvent substitution may pose limitations on analytical performance.

In sum, the works presented in this thesis demonstrated that sustainable analytical chemistry can be advanced through the innovation of existing technologies rather than exclusive reliance on high-end techniques. Both low-pressure chromatography and microextraction workflows were developed to achieve analytical results comparable to conventional methods while reducing solvent consumption, energy demands, and operational complexity.

Despite the promising results obtained, the analytical strategies presented in this thesis can also be considered as frameworks for further developments rather than final solutions. The results presented provide a coherent foundation for further investigation of simplified chromatographic instrumentation and greener analytical methodologies.

Future perspectives for low-pressure chromatographic systems include: assessment of the long-term robustness and stability of low-pressure chromatographic systems to characterize the performance of these systems; evaluation of alternative propulsion systems to overcome limitations associated with peristaltic pumps, particularly medium- and long-term flow rate variations; and design of a confluence, fully compatible with organic solvents, and capable of providing a rugged and reproducible coupling of the detector to the flow system. The exploitation of different and currently available monolithic columns with different stationary chemical compositions (e.g. amine, diol, C_8) may further expand the selectivity and applicability of these systems. The integration of different electrochemical detection strategies can also be a promising advance towards the design of *ad-hoc* analytical systems.

Concerning DLLME technique, future developments should move beyond simple studies upon hazardous solvent substitution. The use of different derivatization strategies in DLLME can be an alternative to expand the applicability range of this approach to additional target-analysis in complex matrices. Advances in DLLME should also move toward developments in task-specific methodologies enabling improved selectivity and extraction efficiency based on environmentally friendly extraction systems. The integration of microextraction techniques into automated and in-line analytical systems, such as flow-based systems, would reduce manual sample handling and increase reproducibility, potentially enabling high-throughput and continuous monitoring applications.

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