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Grau de Química

Treball Final de Grau

Gradient elution HPLC/UV method for the determination of sweeteners in soft drinks.

Determinació per HPLC/UV en mode gradient d'edulcorants en refrescos.

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Universitat de Barcelona

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

Hi ha una força motriu més poderosa que el vapor, l'electricitat i l'energia atòmica: la voluntat.

Albert Einstein

Agraeixo a la Dr. Encarnación Moyano i a la Dr. Clara Ràfols el seu suport constant, les seves ensenyances i l'oportunitat brindada per descubrir com és el món de l'investigació universitaria. De la mateixa manera, agrair als meus pares, amics i companys de feina per ajudar-me en aquest viatge i fer-me'l més lleuger.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The Sustainable Development Goals (SDGs), adopted by the United Nations in 2015, are a set of 17 global interlinked goals that aim to end poverty, protect the planet and improve the prosperity and Peace in the forseable future. The 17 categories can be grouped into five broad areas known as the 5 P: people, planet, prosperity, Peace and partnerships.

This work focuses in the people axis, specifically in SDG 3 on Health and Well-being. As the processed foods are on the rise, health has become an undeniable global concern due to the vast quantity of additives and artificial compounds absorbed by the human body without our previous knowledge. The objective of this TFG is to determine and reveal the usually unknown concentration of artificial sweeteners in soft drinks to raise awareness of the dangerous levels of exposure and induce a much deeper reflection of where the modern diet is headed, as for now it seems to be a rapid, easy and unhealthy approach instead of the diverse, enriching and earthy based that used to be in the past.

Figure I. ODS 3

Understanding the impulses that drive our society to these extremely unhealthy behaviours is the baseline to improve the quality of living and the well-being of the people. Improving such aspects not only will reflect in how we eat but the overall mental health and energy levels will rise which will make easier to do more and to connect more effortlessly between each other that inevitably will create a better world for all of us if like-minded people work regarding to the completion of the SDG envisioned for 2030.

CONTENTS

1. SUMMARY

As the consumption of processed foods with added sweeteners has risen in the recent years and often their amount is not declared, it is of the outmost importance to determine the quantity of additives ingested in our daily routine. High Performance Liquid Chromatography (HPLC) in gradient elution mode is a useful method frequently used in analytical laboratories for such determinations.

Acesulfame-K, aspartame, cyclamate, saccharin, sucralose and neohesperidin dihydrochalcone are among the most used artificial sweeteners in the soft drink industry. The aim of this work is to develop a new UHPLC/UV (Ultra-High Performance Liquid Chromatography with spectrometry detection) method using a gradient elution mode and a reversed-phase C18 column that enables the multi-determination of the aforesaid sweeteners in soft drinks along other common preservatives and antioxidants. This reversed-phase chromatography method is validated in terms of limit of detection, limit of quantification, linearity of the working range, repeatability and reproducibility. To test the applicability of the method, several soft drink samples will be analysed. The final UHPLC/UV method developed will be transferred onto a teaching guide for future undergraduate students of the subject of "Laboratori de Química Analítica, LQA".

Keywords: Gradient elution, Ultra-High Performance Liquid Chromatography with spectrometry detection, UHPLC/UV, acesulfame-K, aspartame, cyclamate, saccharin, sucralose, neohesperidin dihydrochalcone, reversed-phase, C18 column.

2. RESUM

Atès que el consum de menjar processat amb edulcorants afegits ha augmentat en els darrers anys i sovint no es declaren les seves quantitats, existeix la gran necessitat de determinar la quantitat d'additius ingerida a la nostra dieta. La Cromatografia de Líquids d'Elevada Eficàcia (High Performance Liquid Chromatography, HPLC) amb mode d'elució en mode gradient és un mètode d'ús freqüent en els laboratoris analítics per realizar aquestes determinacions.

L'acesulfam-K, l'aspartam, el ciclamat, la sacarina, la sucralosa i la neohesperidina dihidrocalcona són uns dels edulcorants artificials més utilitzats en les begudes refrescants.. L'objectiu d'aquest treball és desenvolupar un nou mètode d'UHPLC/UV (Cromatografia de Líquids d'Elevada Eficàcia) amb elució en gradient i fase inversa amb columna C18 que sigui capaç de dur a terme la multideterminació dels edulcorants esmentats en begudes refrescants, a més a més d'alguns conservants i antioxidants més comuns. El mètode cromatogràfic de fase inversa es validarà mitjançant la determinació límits de detecció, límits de quantificació, linearitat de l'interval de treball, repetibilitat i reproductibilitat. Per a demostrar l'aplicabilitat del mètode, s'analitzaran algunes mostres de begudes refrescants. El mètode òptim es transcriurà en una guia de pràctiques de laboratori pels futurs estudiants de l'assignatura "Laboratori de Química Analítica, LQA"

Paraules clau: Elució en mode gradient, Cromatografia de Líquids d'Elevada Eficàcia amb detecció per espectrometria, mètode UHPLC/UV, acesulfam-K, aspartam, cyclamat, sacarina, sucralosa, neohesperidina dihidrocalcona, fase inversa, columna C18.

3. INTRODUCTION

Sugar has captivated the human palate since the V century AD, when the Gupta Empire, in what is now northern India, discovered how to turn the juices of the sugar cane into the granular crystals called khanda (खण्ड) that we know as sugar. Since then, its popularity has grown exponentially throughout history, making it a key element in our modern diet [1] [2].

Although sweetness originally traces back to nature, the ever-evolving search for the ultimate expression of this cherished flavour has led to the discovery of saccharides and complex sugars, that are chemically produced to create the sensation of sweetness without the caloric burden of natural sugars. These artificial sweeteners range from saccharin and aspartame to sucralose and steviol glycosides.

Low and no calorie sweeteners (LNCS) are by definition classified as artificial non-nutritive and high- intensity sweeteners. There are only nineteen LNCS that have been authorized for consumption in Europe [3] [4] [5]. Even though these additives are substitutes of natural sugars, is not unusual to see LNCS referred by name in the labelling but with no concentration detailed, so people are aware of their presence, yet they are unconscious of their quantities in foodstuffs. Therefore, their main dietary sources might be unclear for the general public[6].

As the consumption of processed foods and beverages with additives has increased in recent decades, sugar consumption has consequently dramatically risen. The food industry emphasizes their positive aspects such as being tooth-friendly, improving the quality of life for different forms of diabetes or allowing weight control in processed foods. However, there is a common concern about the safety of LNCS and the reasons why it is broadly implemented among foods and beverages. Their role as a control management for these high-calorie diets is still controversial, as researchers have found no apparent benefit of their consumption and even weight gain, metabolic syndrome or type 2 diabetes as an adverse effect when consuming a diary [7] [8] [9].

Furthermore, LNCS are present in every aspect of our daily life from diet soft drinks, sport drinks, energy drinks, soya drinks to yogurt and fermented milks [10]. Also, added sugars in chocolates, sports drinks, energy drinks, ice creams, jams, bakery and pastry constitute a major

part of our diet. These facts among the their controversiality are related to the European perspective to reduce sugar consumption through high taxation and strict control of new artificial sweeteners in order to achieve the ODS Goal 3: Good Health and Well-being by 2030, as envisaged by the United Nations in 2015.

3.1. EUROPEAN LEGISLATION

In the European Union (UE), there are only six artificial high-intensity sweeteners approved from the aforementioned LNCS, which include acesulfame-K (ACS-K), aspartame (ASP), sucralose (SCL), saccharin (SAC) and its salts, cyclamate (CYC) and neohesperidin dihydrochalcone (NHDC). Those UE sweeteners are safety assessed by the European Food Safety Authority (EFSA) prior authorization. EU directives 94/35/EC, 96-83/EC, 2003/115/EC, 2006/52/EC define the regulations in the European region [11]. The USA, for example, does not include cyclamates and neohesperidin instead the use of neotame is accepted.

3.1.1. European Denomination, Code E

The sweeteners are noun with the same code E-XXX used for other additives as well. The scheme for its numeration is set by the International Numeration System (INS) established by the Alimentarius Codex and regulated by EFSA. The sweeteners ranged from E-950 (ACS-K) to E-969 (advantame). Currently UE approved sweeteners (used as table-top or additives) based on Regulation Number 129/2011 of the European Commission of November 11, 2011 can be seen in Table 1.

Table 1. UE approved sweeteners with maximum dosage permitted in soft drinks. **quantum satis* is a Latin expression that refers to a correct use: sufficient to achieve the objective but not superior than the necessary. Taumatine is not used in the soft drink industry.

Sweetener	Noun	Maximum dosatge in soft drinks (mg/L)
Sorbitol	E-420	*quantum satis*
Manitol	E-421	*quantum satis*
Acesulfame-K	E-950	350
Aspartame	E-951	600
Cyclamate	E-952	250
Isomaltose	E-953	*quantum satis*
Saccharin	E-954	80

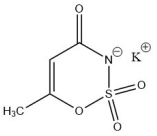
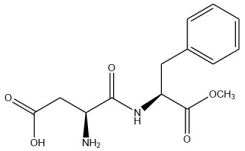
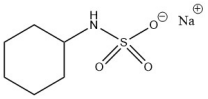
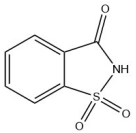
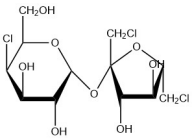
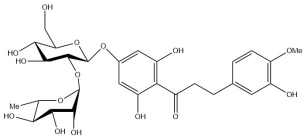
Sucralose	E-955	300
Taumatine	E-957	----
Neohesperidin DC	E-959	30
Neotame	E-961	20
ACS-K / ASP salts	E-962	250
Malitol	E-965	*quantum satis*
Lactitol	E-966	*quantum satis*
Xylitol	E-967	*quantum satis*

3.2. ARTIFICIAL SWEETENERS

High-intensity sweeteners can be divided in three categories: natural, synthetic and semi-synthetic. Although there is an extensive diversity of organic compounds like carbohydrate derivatives, salts of organic acids, terpenoids or protein, the majority of approved sweeteners are artificially created [12]. The most popular due to their physicochemical properties are ACS-K, ASP, CYC, SAC, SCL, alitame, neotame and NHDC that is semi-synthetic.

The additives can be used separately or in combination with others making it a blend of sweeteners. Blends are used as the prime solution because some sweeteners can complement the aftertastes of others and a mixture offers a better sophisticated taste [13]. Blending not only eliminates the undesired aftertastes but sometimes results in a greater increment of sweetness than the algebraic sum of the components, a common example is the SAC-CYC (1:10) mixture. Moreover, a tailored sweetener blend is more capable of reproducing the texture and sweetness profile of sugar-containing products. It is worth mentioning that in the EU these additives are mainly available in salt form, usually sodium salts. Table 2. shows relevant information of the most popular sweeteners that will be the object of study.

Table 2. Structure and physiochemical properties of the studied sweeteners. Information extracted of HMDB. *Sweetness Strength is referred by comparison to a standard sucrose solution.

Sweetener	Structure	Molecular Weight (g/mol)	pka	logP	Water solubility (g/L)	Sweetness Power*
Acesulfame-K		201.24	2.20	-1.514	11.42	200
Aspartame		294.3	3.01	-0.077	0.65	133
Cyclamate		201.2	9.57	0.319	7.68	35
Saccharin		183.2	1.60	0.452	6.51	450
Sucralose		397.63	12.52	-0.734	12.91	650
Neohesperidin DC		612.57	9.56	-0.091	0.34	1500

3.3. ANALYTICAL METHODS

Analytical methods that include high performance liquid chromatography (HPLC) (reversed-phase and ion chromatography), thin layer chromatography, gas chromatography, flow injection analysis, capillary electrophoresis, electro-analysis and spectroscopy are able to determine the concentration of sweeteners individually and/or simultaneously in mixtures as can be seen in Fig. 1.

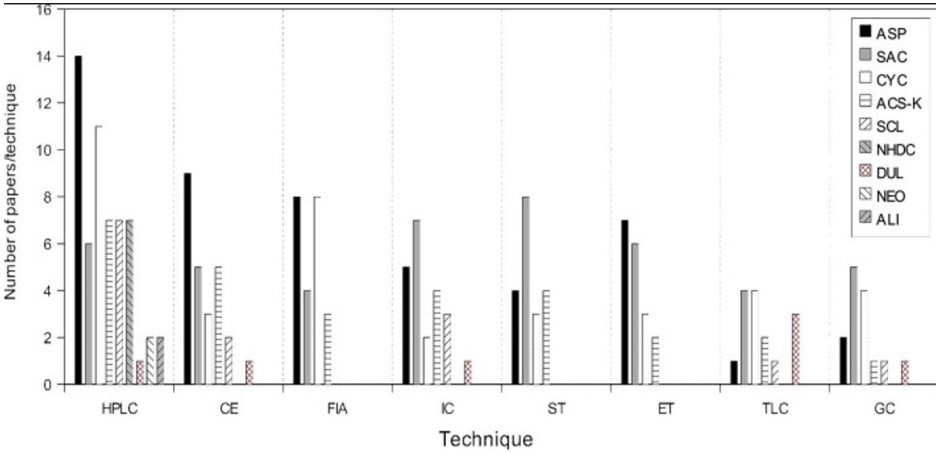


Figure 1. Analytical techniques for determination of artificial sweeteners. Capillary electrophoresis, Electroanalytical techniques, Flow-injection analysis, Gas chromatography, Reversed-phase HPLC, Ion chromatography, Thin-layer chromatography and Spectroscopic techniques. [14]

Since there are multiple possible combinations for the addition of sweeteners in food, methods have the necessity of being able to perform a multi-determination in the same run. To a certain extent, these methods focus on the determination of only 3-4 sweeteners although recently, some progress has been made for methods covering a much broader range of additives [15] [16]. Table 3 contains references to recently published multi-analyte methods for the determination of artificial LNCS.

Table 3. HPLC-UV analytical procedures for simultaneous determination of artificial sweeteners mixtures in samples of different food products

Analyte	Sample	Mobile phase	Column	Analytical parameters	Ref
ACS-K, ASP, SAC, benzoic acid (BEN), sorbic acid, tartrazine	Soft drinks	MeOH – phosphate buffer (pH 4)	Lichrosorm C18 (250 x 4.6 mm 10 µm)	Recovery: 98.6-102.3% LOD: 0.1-3 mg/L	[17]
ACS-K, ASP, SAC, (BEN), sorbic acid	Beverages, jams	MeOH – phosphate buffer (pH 6)	Spherosorb ODS 1-C18 (250 mm x 4.6 mm x 10 µm)	Recovery: 98.1-104.2% LOD< 0.1 mg/L	[18]
ACS-K, ASP, SAC, (BEN), sorbic acid, vanillin	Cola and instant powder drinks	ACN – $NH_4CH_3CO_2$ (pH 4)	YMC ODS Pack AM (250 mm x 4 mm x 5 µm)	Recovery: 99-101% LOD: 0.2-3.1 10 µg/g	[19]

ASP, SAC	Dietary products	0.08 M TEA-phosphate buffer (pH 3) – MeOH - THF	Hypersil C-18 (250 mm x 4 mm x 10 µm)	Recovery: 95-97% LOD: not available	[20]
Alitame, ASP, neotame	Various foods	ACN – phosphate buffer (pH 4)	Cosmosil 5C18-AR	Recovery: 89-104% LOD: 1 µg/g	[21]

Of the extensive range of methods, the chromatographic receive wide recognition. From all of them, HPLC has become dominant in the field due to the massive availability of detectors and different mechanisms of separation that endow its universal character. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is perfectly capable for the separation and determination of artificial sweeteners.

Albeit HPLC is the leading technique, other separation methods have also found applications in this area. Ion chromatography is suitable for multi-analysis in food products and more advantageous than RP-HPLC in terms of cost and Green Chemistry for certain analytes [22]. Capillary electrophoresis is another interesting technique for multi-determination as in terms of separation power, solvent consumption and analysis time might be superior to HPLC in some cases [23]. Other techniques such as flow-injection [24], spectroscopy [25] and electrochemical methods [26] are also used for the single determination which allow for a rapid, reproducible and simpler analytical methodology.

3.3.1. Reversed-Phase High Performance Liquid Chromatography

RP-HPLC methodologies are based on isocratic or gradient chromatographic separation and a wide range of detection systems can be implemented: ultraviolet-visible (UV/vis), amperometric, coulometric, spectrofluorometric, mass spectrophotometry (MS), conductometric and light scattering detectors.

Artificial sweeteners containing chromophore groups like ASP, NHDC, ACS-K and SAC are usually analyzed via a UV/vis detector since the functional group give a signal response in a high selective wavelength [17]. Alternative, a diode array detector could be used to monitor an extensive spectrum of wavelengths [27]. Lacking chromophore sweeteners such as CYC and SCL can be derivatized to introduce a chromophore group in their structure, as an example, by treating SCL with p-nitrobenzyl chloride (PNBCl) a strong UV-absorbing derivative at 260 nm is

produced [28]. On the other hand, cyclamate can be acidified to cyclamic acid which could be converted to N,N-dichlorocyclohexylaamine [29] and its posterior derivatization with 4-fluor-7-nitrobenzofurane (NBD-F) produced a high absorption compound at 485 nm [30].

As the majority of EU approved sweeteners can be ionizable either positive (CYC, NHDC, SAC, ASP, ACS-K and glycine) or negatively charged (advantame, alitame and neotame). They are suitable for analysis by HPLC-MS or HPLC-MS/MS. An extremely high selective technique capable of separating each component even in complex matrices, that fact enables a multi-determination up to 12 different sweeteners [31].

In RP-HPLC, mobile phases are normally composed of an aqueous solution and an organic solvent (see Table 3). Acetonitrile and methanol are among the most used organic solvents, although the choice depends on the column and analyte determined, acetonitrile is the common solvent of choice. Phosphate buffers are the prevalent aqueous solution but other buffers such as triethanolamine or ammonium acetate are also relevant for these determinations.

Typically, the determination of artificially sweeteners by RP-HPLC are done in gradient elution mode. Due to the different hydrophilic character of the diverse sweeteners, the gradient elution mode permits the possibility of a multi-determination of analytes in the same run, with reduced time analysis and solvent waste. Alternatively, isocratic elution mode is used more regularly if a single analyte determination is done as the working conditions are optimised uniquely for that specific sweetener.

3.3.2. Sample Treatment

A previous sample treatment before HPLC analysis is an essential part of the analytical process because food samples are among the most difficult matrices due to their variability and complex compositions. This stage must be designed specifically for a given method considering the procedure and instrumentation involved. Generally, the difficulty in the sample treatment consist in the co-extraction of components with the analytes as a result of similar solubility and matrix interferences that could generate emulsions, turbidity or masking of the analyte. Clean up of the extract by means of dialysis, liquid-liquid extraction, precipitation, filtration and stationary phase are some of the most used solutions. Such procedures not only are analytically important, but if they are optimally applied, analysis time will be reduced.

There are very scarce methods for sweeteners requiring no sample treatment like electrochemical [26] and FTIR spectrometry-based methods [32]. However, a time-consuming sample-specific calibration is required and that is their major drawback.

The complexity of the sample treatment depends on the matrix of the sample. Relatively simple matrix composed of table-top solid and liquid sweeteners, beverages, powdered drinks, syrups and juices can be diluted in water, organic solvents or a buffer mixture. Carbonated drinks must to be degassed prior the analysis mainly by sonication, but vacuum or nitrogen sparging can be other options. The samples can be clarified if desired, which is useful for the elimination of proteins and suspended particles by occlusion. Samples need to be filtered before its determination, typically a single step filtration with a membrane filter is sufficient, in some cases, centrifugation or pre-filtration with filter paper might be added steps. Filtration treatments are widely used although no chemical interferences with a good water solubility are removed and no preconcentration is achieved.

Solid phase extraction (SPE) is a more sophisticated treatment technique that separates the components of the matrix in different groups based on their affinity to a certain compound of the stationary phase. There is an enormous number of types of SPE cartridges available, non-polar C18 is the prime contender in the market, but polar packing materials, polystyrene, dextran and other polymer fillings are also used [33]. This method allows the elimination of most interferences in the more complex matrices samples.

In SPE procedures, cartridges are conditioned, afterwards the samples are loaded and cartridge can be washed before the elution of analytes with an appropriated solvent. Interferent compounds that have less interaction with the adsorbent are weakly retained and easily separated from analytes that should show a stronger interaction with the adsorbent selected. Although, the procedure can undergo oppositely and analytes be less retained than interferences, then the SPE is used as a washing procedure [34]. Evaporation to dryness of the final SPE extract and its reconstitution with a smaller portion of a selected solvent enhance the sensitivity of the determination due to preconcentration factor.

4. OBJECTIVES

The general objective of this TFG is the development of a UHPLC/UV-vis method using gradient elution mode and reversed-phase C18 column for the determination of artificial sweeteners in commercial soft drinks. To achieve this main objective, the follow specific objectives should be accomplished:

- The optimization of the chromatographic conditions to enable a suitable separation of the selected analytes.
- The optimization of the detection conditions to maximize the signal and to achieve the necessary detectability and selectivity.
- The validation of the developed RP-UHPLC/UV-vis method by quality parameters such as limit of detection, limit of quantification, linearity of the working range, repeatability and reproducibility.
- The analysis of soft drink samples to test the applicability of the developed method.
- The transfer of the final optimized and validated RP-UHPLC/UV-vis method onto a teaching guide for its use by future undergraduate students of the subject “Laboratori de Química Analítica, LQA”.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Reagents and Chemicals

The chemicals used to prepare the stock standard solutions and the solvents involved in the procedure are listed below. Note that the chemicals were employed without further purification and used as received.

5.1.1.1. Standards

- Acesulfame-K from Supelco Analytical, USA.
- Aspartame from Supelco Analytical, USA.
- Benzoic acid from Probus, Spain.
- Caffeine 98.-101% from Panreac, Spain.
- D-(+)-Sucrose from Chimica, Spain.
- D-(-)-Fructose from E-Merck, Darmstadt, Germany.
- L-(+)-Ascorbic acid 99-100.5% from Analar Normapur, Belgium.
- Neohesperidin dihydrochalcone ≥ 95% from Sigma-Aldrich, USA.
- Quinine hemisulfate monohydrate suitable for fluorescence ≥ 95% from Sigma-Aldrich, USA.
- Saccharine from Prolabo, Spain.
- Sodium di-Hydrogen Phosphate 2-hydrate from Panreac, Spain.
- Sucralose powder from Sigma-Aldrich, USA.

5.1.1.2. Solvents

- HPLC water grade obtained by ultrapurification using a Milli-Q water purification system (Merck, Spain).
- Hydrochloric Acid 37% (Reag. USP) for analysis, ACS, ISO from Panreac, Spain.
- Methanol for HPLC ≥ 99.9% from Honeywell Riedel-de Haën, Germany.

5.2 Instruments and other Equipment

The apparatus and instruments used for the sample treatment, to prepare the mobile phase and the stock standard solutions were the following:

- Analytical balance Kern ABS 200-4N, precision: 0.0001g.
- Analytical balance Mettler-Toledo AG425, precision: 0.0001g.
- Diverse volumetric glassware of different volumes, Pasteur pipette, volumetric flasks, beakers, and vials.
- Elmasonic S60 sonicator.

- Filtration system for samples and mobile phase: manual plastic syringes for sample filtration and glass system for mobile phase filtration, both using Nylon membrane filters of 13mm (samples) and 45 mm (mobile phase) of 0.45 µm porous size.
- Milli-Q water purification system.
- pH-Meter Crison MicropH 2000.
- Rotilabo mikroliterpipette 100-1000 µL.
- Selecta centrifuge model Centronic-95.
- SGE Microsyringe 100 µL for HPLC.
- UV-Vis Agillent CARY 60 spectrophotometer.

5.3 Liquid Chromatography System

An Agilent 1260 Infinity II System which included a quaternary pump (G7111B), an online vacuum degasser, a variable wavelength spectrophotometric detection system (G7114A), and a Rheodyne manual injector valve are the ones utilized for the experimental section.

5.3.1. Working conditions for the determination of artificial sweeteners

After the optimization described in the Results and Discussion section, the final working conditions proposed for the RP-UHPLC/UV-vis method to multi-determine the analytes are the following:

- Column: InfinityLab Poroshell 120 EC-C18 (100 mm length x 4.6 mm ID x 2.7 µm superficially porous particle size) from Agilent.
- Precolumn: UHPLC InfinityLab Poroshell 20 EC-C18 (5 mm length x 4.6 mm ID x 2.7 µm superficially porous particle size) from Agilent.
- Mobile phase:
    - Solvent (A): aqueous 0.1M phosphate buffer at pH=4.00 prepared from NaH_2PO_4, the pH was adjusted by dropwise addition of HCl until the desired value (usually one or two drops were needed for 500 mL of solution).
    - Solvent (B): methanol.
    - Solvent A and B were filtered with a Nylon membrane of 0,45 µm before their use.
- Elution mode: gradient elution was used as indicated in Table 4.
- Detection: UV-Vis Agilent variable ultraviolet-visible wavelength detection system was used to monitor at 240 nm and 270 nm.
- Flow rate: 1 mL/min.
- Injection volume: 10 µL.
- Column temperature: at room temperature.

Table 4. Elution program of the mobile phase.

Time (min)	A (%)	B (%)
0.00	85.0	15.0
1.00	85.0	15.0
4.00	20.0	80.0
7.00	20.0	80.0
15.00	85.0	15.0
16.00	85.0	15.0

5.4 Standard Preparation

Since all the sweeteners, compounds and preservatives studied are soluble in water, separate stock standard solutions for quantitative analysis were prepared by dissolving each compound in Milli-Q grade water at a concentration of 100 mg/L. Additionally, mixed stock standard solutions containing all the additives were used to optimize the working conditions. For quantitative analysis, the stock standard solutions were prepared at concentrations of 500, 100, 80, 60, 40, 20, 10 and 4 mg/L equally for each analyte. Before the analysis, the samples were filtered through a 0.45 µm Nylon membrane syringe filter.

5.5 Sample Preparation

Soft drinks samples were purchased from local supermarkets and preliminary degassed by ultrasonication by soaking in for 10 minutes. Prior to the analysis, the samples were diluted with HPLC water grade if necessary and filtered through a 0.45 µm Nylon membrane filter. Cola drinks were diluted 10/25, Fanta beverages were diluted 5/50 and tonic drinks were directly injected without dilution. Additionally, Fanta samples had to be centrifugated at 3000 rpm during 10 minutes before their filtration with the syringe filter since they contain juices extracts.

6. HPLC METHODOLOGY FOR LNCS ANALYSIS

6.1 Wavelength optimization

The UV-vis spectrum of each additive and the mobile phase was obtained using UV-Vis Agilent CARY 60 to select the optimum wavelength used during the chromatographic separation. As expected the mobile phase absorption was negligible in the range explored 190 nm to 600 nm.

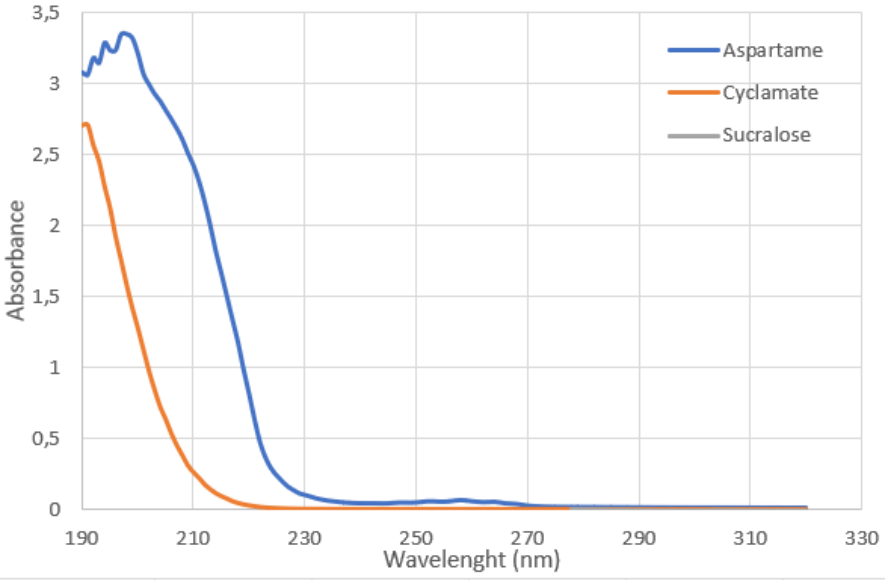


Figure 2. Representative spectrum of standard LNCS in soft drinks measured by Agilent 1260 Infinity II LC System. LNCS: Aspartame, Cyclamate, Sucralose. Detection range: 190-320 nm.

CYC and SCL are LNCS that did not show absorption in the UV-vis range as could be seen in Fig. 2, so they could not be included in this TFG via direct analysis by UHPLC-UV/vis. However as mentioned before, various published methods proposed few derivatization procedures for their determination by RP-HPLC with UV-vis or fluorimetry [28] [29] [30]. Such methodologies convey a different analytical route for their suitable determination that could be implemented in the LQA student lab in the future.

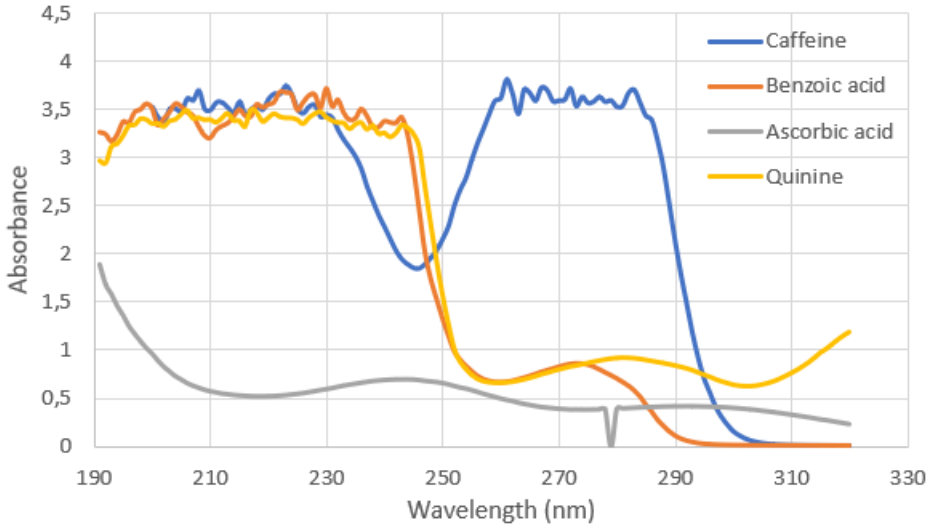


Figure 3. Representative spectrum of common additives in soft drinks measured by Agilent 1260 Infinity II LC System. Additives: Caffeine, Benzoic acid, Ascorbic acid, Quinine. Detection range: 190-320 nm.

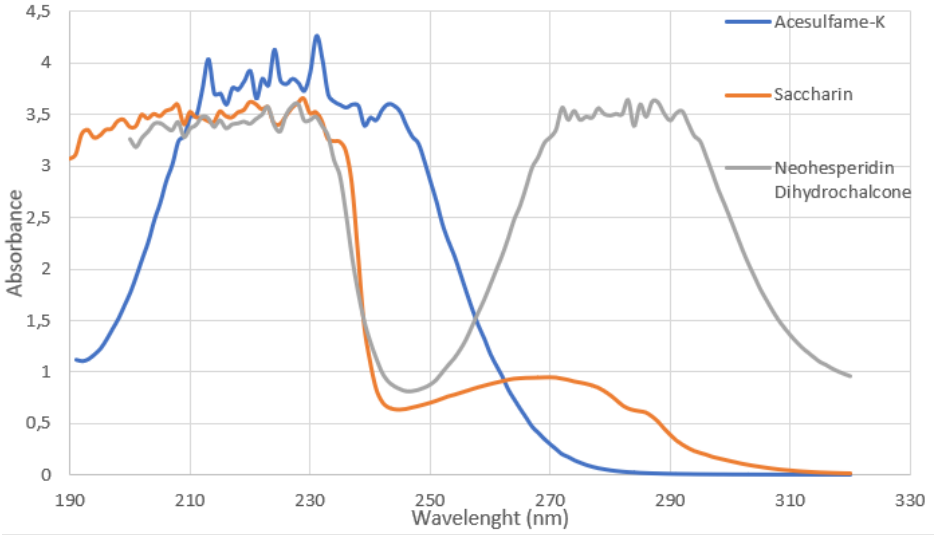


Figure 4. Representative spectrum of standard LNCS measured by Agilent 1260 Infinity II LC System. LNCS: Acesulfame-K, Saccharin, Neohesperidin dihydrochalcone. Detection range: 190-320 nm.

As observed in the Fig. 2, Fig. 3 and Fig. 4 the sweeteners and other common additives absorb within a wide range of wavelengths. ASP has its maximum absorption at 210 nm, while, quinine (QUIN) shows maximums from 200 to 250 nm, from 270 to 290 nm and from 320 to 330 nm, ACS-K absorbs between 220 and 230 nm and; NHDC, SAC, caffeine (CAF) and acid benzoic (BEN) have two absorption ranges, one between 210 to 230 nm and the other between 260 to 280 nm.

The initial analyses were performed monitoring at 210 nm to include ASP as is present in the majority of the samples. However, at this wavelength, the sweeteners showed poor results with the selected multi-determination gradient which is indicated in Table 4 due to high background signal that difficult its determination. For that reason, the monitorization at 210 nm was discarded in this study.

As an alternative method for future studies, the introduction of a chromophore group via derivatization allowing the detection of ASP at higher wavelengths could be investigated. As an example, the derivatization of its hydrolysis products (asparthylphenylalanine, aspartic acid and phenylamine) by reaction with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion in a mildly alkaline medium give a highly fluorescent 1-cyano-2-substituted benz[f]isoindole derivative [35] that can be determine at a highly selective wavelength avoiding the existent difficulties.

Since the detection system used allows the data acquisition in a wavelength switching mode, the optimal wavelengths that can be proposed are 240 and 270 nm, in which all the compounds (except ASP) can be easily detected without interferences, enabling the multi-determination of various analytes with better sensitivities.

6.2 Chromatographic Separation

The selected Agilent Poroshell 120 EC-C18 column used is suitable for the separation of all additives studied, since they have reasonably pronounced non-polar character due to the aromatic ring or aliphatic chains present in their structures. A moderate acidic mobile phase (pH 4.00) was chosen as Solvent A to minimize the analyte ionization (see pKa values in Table 2) and favor the retention of the hydrophobic stationary phase (C18) [17]. The 0.1M phosphate buffer was selected owing to its great versatility and no absorption in a wide range of wavelengths, diverse published methods were able to analyze artificial sweeteners, preservatives, antioxidants,

dyes and alkaloids in various samples matrices using this buffer. Furthermore, the buffer can be applied in diverse techniques other than HPLC-UV [36] [37]. Methanol has been selected as the organic modifier, because it is a suitable and cheap organic solvent, instead of acetonitrile, a more common and usually preferred choice by the industry [38] [39][40]. It is acknowledged that the method will be used in a practice for laboratory students and ratio cost to solvent performance has been borne in mind.

The individual stock standard solutions were injected under isocratic conditions to study the retention behavior of each sweetener. These retention times and partition coefficients shown at Table 2 were considered to select the gradient elution conditions that after numerous attempts of different compositions, a final gradient elution program showed in Table 4 was proposed. Which simultaneously reduces analysis time and allow maximum separation (resolution) between analytes. It must be considered that under isocratic conditions, the analysis time would increase up to 30 minutes and this is unacceptably long.

The percentage of organic modifier (Solvent B) and the schedule for the gradient elution program Table 4 were optimized. A higher percentage of aqueous solution (Solvent A) is used in the first isocratic stage to allow the separation of more polar sweeteners from the elution front by favoring their retention on the C18 stationary phase. The linear gradient elution allows the elution of the different compounds with enough chromatographic resolution and a maximum of 85% of organic modifier (Solvent B) was set in order to avoid precipitation of phosphates.

According to the equation of Van Deemter Eq. 1, when the diameter of packed particles decreases, the Eddy-diffusion parameter (term A) decreases. Therefore, the theoretical height of the plates (H) decreases and this provides a much larger number of plates (term N) for a similar column length. The Poroshell column, packed with superficially porous particles (2.7 μm), shows higher separation efficiency (lower H) than the totally porous particles of the same size. The optimal flow rate of the mobile phase depends on the internal diameter (ID) of the column. For a 4.6 mm ID, the optimal flow-rate is 1 mL/min.

$$H = A + \frac{B}{u} + Cu \qquad N = \frac{L}{H}$$

Equation 1. Equation of Van Deemter, term H: height of plates, term A: Eddy-diffusion parameter, Term B: longitudinal diffusion coefficient, term C: mass transference coefficient, term u: lineal velocity. Term N: number of plates

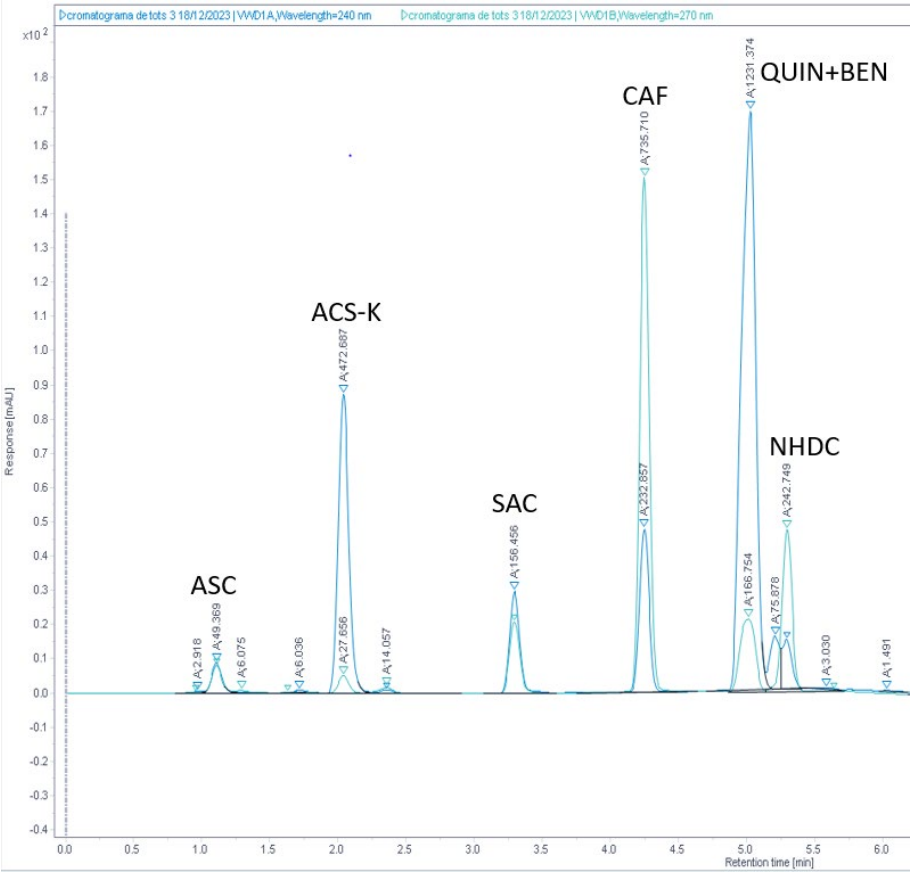


Figure 5. Representative RP-UHPLC/UV-vis chromatogram of sweeteners and additives. Sweeteners: Acesulfame-k (ACS-K), saccharin (SAC), neohesperidin DC (NHDC). Additives: ascorbic acid (ASC), caffeine (CAF), quinine (QUIN), benzoic acid (BEN); Mobile phase: methanol/phosphate buffer gradient elution; Column: Poroshell 120 EC-C18 100 mm x 4.6 mm x 2.7 µm; Detection range: 240 nm, 270 nm; Flow rate: 1 mL/min; Injected volume: 10 µL.

The chromatographic separation is shown in Fig. 5, where it can be observed that the peaks are well resolved at baseline, meaning that a good resolution and sufficient separation between consecutive peaks is achieved. Moreover, chromatographic peaks also show a good shape (gaussian and symmetric shape) indicating the good performance of the chromatographic

method. The high hydrophilicity of ascorbic acid (ASC) obliges to a high aqueous initial mobile phase for its retention. However, the high hydrophobicity of NHDC made necessary the use of a mobile phase with higher elution strength, so a high percentage of methanol is needed to elute this compound see Table 4. Each gradient elution program finishes with an isocratic step at high percentage of methanol (from 7 to 15 minutes) to clean the column and a final turning back to initial conditions in a short time. The final stage is an isocratic step of 1 minute that allows the stabilization of the column and conditions it for the next injection.

Although, QUIN and BEN coelute at 5 minutes does not suppose a major issue for the objective of this TFG because BEN is not present in any of the samples analyzed in this TFG and it was just injected since is a commonly used preservative. If BEN was identified in any sample, the separation should be reoptimized. These final enhancements led to an unequivocal determination of the distinct analytes and the verification of the optimized gradient meaning that the working conditions are suitable for a multi-determination of high intensity artificial sweeteners (ACS-K, NHDC and SAC) including some of the most common additives like antioxidants (ASC) and pervasive compounds in the soft drink niche like CAF or QUIN.

7. METHOD VALIDATION

The RP-HPLC/UV-vis method proposed has been validated. Different quality parameters such limit of detection (LOD), limit of quantification (LOQ), linearity, reproducibility, and repeatability have been evaluated.

7.1 Limit of detection, limit of quantification

LOD and LOQ were estimated to evaluate detectability of the RP-UHPLC/UV-vis method. These limits were determined by injecting solutions at a low concentration level and based on a signal-to-noise ratio of 3:1 for the LOD and 10:1 for the LOQ. The results are shown in Table 5. In general, LODs range from 0.23-1.23 mg/L for all the additives, the lowest was caffeine (CAF) and the highest ascorbic acid (ASC). The limits of quantification (LOQs) were in the range 0.76-4.10 mg/L.

Table 5. LODs and LOQs estimated for the studied analytes.

Compound	LOD (mg/L)	LOQ (mg/L)
ACS-K	0.69	2.28
ASC	1.23	4.10
BEN	1.03	3.43
CAF	0.23	0.76
NHDC	1.14	3.81
QUIN	0.63	2.09
SAC	1.01	3.38

7.2 Linearity of the working range

Linearity was studied for ACS-K and NHDC, which were the sweeteners identified in the samples and must be quantified. Standard solutions with concentration levels ranging from 4 mg/L (the LOQ) to 100 mg/L (the higher concentration to avoid saturation of the system) and shown in Table 6 were injected. Fig. 6 shows, as an example, two calibration curves obtained during the validation study and Table 7 shows the results of the regression analysis for the calibration curves. The calibration standards were injected several times every day to study repeatability and reproducibility as well as for the quantification of the different samples. As can be seen in the results showed (Table 7), the analytes in the RP-UHPLC-UV/vis method were linear and reproducible within the concentration working range studied providing correlation coefficients (R^2) better than 0.9918 and the calibration curves were reproducible between days since lower values of RSD for the slope were calculated.

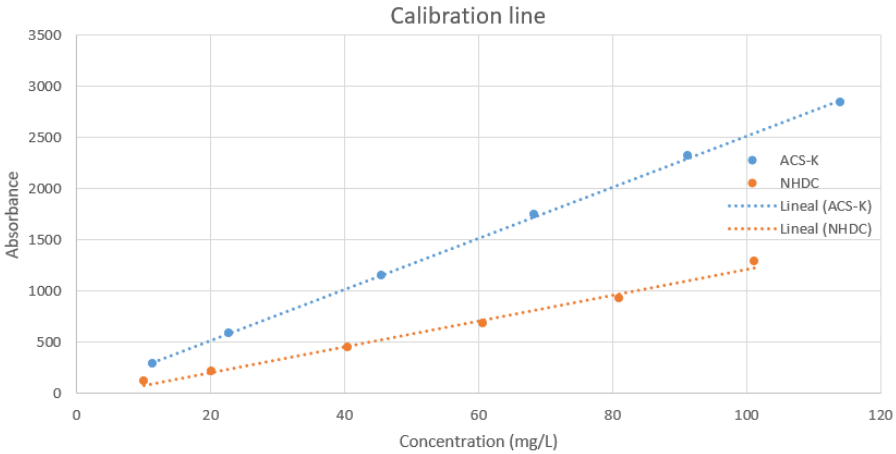


Figure 6. Representative graphic of the calibration curves of acesulfame-K (ACS-K) and neohesperidin dihydrochalcone (NHDC).

Table 6. Concentration range of calibration curves of the sweeteners studied.

Sweeteners	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
ACS-K	4.6	11.4	22.8	45.6	68.4	91.1	114
NHDC	4.0	10.1	20.2	49.5	49.5	80.9	101

Table 7. Calibration curves estimated for the studied analytes in two different days.

Compound	Conc. range (mg/L)	Slope	Intercept	Correlation coeff. (R^2)
ACS-K (Day1)	11.4 - 114	25.1	13.6	0.9997
ACS-K (Day2)	4.6 - 114	25.1	5.8	0.9998
NHDC (Day1)	10.1 – 101.2	12.6	-42.5	0.9918
NHDC (Day2)	4.0 – 101.2	12.5	-24.1	0.9937
ACS-K RSD (%)	---	0.0098		---
NHDC RSD (%)	---	0.58		---

7.3 Repeatability and Reproducibility

To evaluate the method variability, both repeatability and reproducibility were estimated. Repeatability as the variation of short-term measurements, was evaluated by injecting by triplicate the same standard on the same day, the results are expressed as the average value of the 3 replicate injections and its relative standard deviation (RSD%). While reproducibility as the variability in long-term measurements was estimated by analysing the same standard in 3 different days. The results are reported as the average value of 3 replicates in 3 independent days and the corresponding RSD%. Additionally, the accuracy was estimated by comparing the calculated concentration using the calibration curve in front of the real concentration of the standard (see Table 8 and Table 9). As can be seen, great accuracy levels are shown for ACS-K between 98.9% and 100.9%, NHDC, except in level 1 where the error is considerably larger, probably because this level is too close to the LOQ level.

Table 8. Concentration, retention time, area, back-calculated concentration and accuracy of calibration curves of acesulfame-K.

ACS-K				
Conc. (mg/L)	**t_R (min)**	**Area**	**Back. Calc. Conc. (mg/L)**	**Accuracy (%)**
114	1.980	2848.5	113	100.6
91.1	1.982	2320.1	92.3	98.9
68.4	1.982	1721.2	68.4	100.4
45.6	1.992	1140.7	45.2	100.5
22.8	1.975	580.8	22.9	99.5
11.4	1.978	291.0	11.3	100.3
4.6	1.998	119.2	4.50	100.9

Table 9. Concentration, retention time, area, back-calculated concentration and accuracy of calibration curves of neohesperidin dihydrochalcone.

NHDC				
Conc. (mg/L)	**tR (min)**	**Area**	**Back. Calc. Conc. (mg/L)**	**Accuracy (%)**
101	5.267	1296.9	106.0	95.5
80.9	5.268	934.1	76.9	105.3
60.7	5.264	701.2	58.2	104.4
49.5	5.265	482.8	41.7	99.5
20.2	5.262	212.2	18.9	106.7
10.1	5.268	115.7	11.2	90.21
4.00	5.268	48.9	5.9	69.0

Table 10, Table 11 and Table 12 show the results obtained during the repeatability study. As can be seen, the relative standard deviation obtained for the peak area, the corresponding retention time, and the concentration calculated by interpolation in the calibration curve for each compound were lower than 1.87%, 4.43%, and 1.88% for ACS-K and 5.67%, 0.190% and 5.39% for NHDC respectively. Which indicates that the method is repeatable.

Table 10. Results of the repeatability of the HPLC-UV/vis method. Day 1, Standard of 40 mg/L.

	ACS-K			NHDC		
	t_R (min)	Area	Conc. (mg/L)	t_R (min)	Area	Conc. (mg/L)
Injection 1	2.053	1165.6	39.8	5.244	454.8	39.6
Injection 2	2.077	1162.4	39.7	5.245	462.5	40.2
Injection 3	1.912	1176.8	40.2	5.241	462.3	40.2
Average	2.014	1168.3	39.9	5.243	459.1	40.0
S	0.089	7.5	0.3	0.002	3.9	0.4
RSD (%)	4.43	0.64	0.65	0.04	0.85	0.96

Table 11. Results of the repeatability of the HPLC-UV/vis method., Day 2, Standard of 40 mg/L.

	ACS-K			NHDC		
	t_R (min)	Area	Conc. (mg/L)	t_R (min)	Area	Conc. (mg/L)
Injection 1	1.963	1148.9	45.3	5.249	448.3	39.1
Injection 2	1.965	1160.2	45.8	5.249	457.6	39.8
Injection 3	1.964	1176.0	46.4	5.249	458.8	39.9
Average	1.964	1160.2	45.8	5.249	457.6	39.6
S	0.001	13.6	0.5	0.000	5.7	0.5
RSD (%)	0.051	1.18	1.18	0.00	1.26	1.38

Table 12. Results of the repeatability of the HPLC-UV/vis method., Day 3, Standard of 40 mg/L.

	ACS-K			NHDC		
	t_R (min)	Area	Conc. (mg/L)	t_R (min)	Area	Conc. (mg/L)
Injection 1	1.992	1140.7	45.2	5.265	482.8	40.7
Injection 2	1.989	1134.2	45.0	5.263	450.0	38.0
Injection 3	1.989	1101.4	43.7	5.264	431.9	38.4
Average	1.99	1135.4	44.6	5.264	454.9	38.4
S	1.732	1125.4	44.6	5.264	25.8	2.1
RSD (%)	0.087	1.87	1.88	0.190	5.67	5.39

As for the reproducibility displayed in Table 13, the relative standard deviation obtained from the average of the 3 independent days shows that area, time retention and concentration for NHDC are reproducible since their RDSs are lower than 3%. ACS-K concentration is also reproducible, but showed a greater variability than NHDC although it is not significantly high (7.40%). Overall, the method is reproducible, and as expected, the variability in repeatability is minor than the reproducibility since their RSD for all the parameters are smaller.

Table 13. Results corresponding to the reproducibility of the HPLC-UV/vis method, Standard of 40 mg/L.

	ACS-K			NHDC		
	t_R (min)	Area	Conc. (mg/L)	t_R (min)	Area	Conc. (mg/L)
Day 1	2.014	1165.6	39.8	5.243	462.3	40.0
Day 2	1.964	1160.2	45.8	5.249	457.6	39.6
Day 3	1.990	1134.2	43.5	5.264	450.0	38.0
Average	1.989	1153.3	43.5	5.252	456.7	39.3
S	0.025	16.7	3.2	0.011	6.2	1.1
RSD (%)	1.25	1.45	7.40	0.206	1.35	2.7

8. SAMPLE ANALYSIS

Since the scope of this TFG is the analysis of artificial sweeteners in soft drinks, a few of the most representative brands were selected. Coca-Cola, Fanta, Pepsi and Schweppes tonic were chosen for their predominant market share and relatively easy matrices. Other samples like Trina were tried as feasible candidates, but sample preparation was found troublesome since its matrix is more complex as it includes juices extracts and acacia gum, a natural polysaccharide that made the detection of the LNCS impossible even though was undergone in a more extensive sample treatment than the other samples. Future enhancement by SPE or similar techniques will be needed for the analysis of such samples. Fanta also shows a slightly more complex matrix than tonics or cola drinks due to the citric juice extracts, however an additional centrifugation step in

the sample treatment was able to address the obstacle. Table 14 and Table 15 show the results obtained and Fig. 7-11 the chromatograms obtained.

Table 14. Results corresponding to determination of ACS-K from soft drinks samples

Sample	ACS-K					
	Injection 1 (mg/L)	Injection 2 (mg/L)	Injection 3 (mg/L)	Average	s	RSD (%)
Coca-cola Light	143.6	146.2	146.2	144.9	1.5	1.05
Coca-Cola Zero	142.0	143.3	142.0	142.6	0.5	0.53
Orange Fanta	225.7	229.1	224.6	225.7	1.0	1.04
Lemon Fanta	211.6	215.0	216.2	215.0	1.1	1.11
Pepsi	90.7	85.6	85.4	88.2	3.4	3.41
Schweeppes tonic	115.5	116.7	115.7	115.7	0.6	0.56

Table 15. Results corresponding to the determination of NHDC from Fanta soft drinks.

Sample	NHDC					
	Injection 1 (mg/L)	Injection 2 (mg/L)	Injection 3 (mg/L)	Average	s	RSD (%)
Orange Fanta	1689.5	1706.3	1690.2	1689.9	9.5	0.56
Lemon Fanta	1584.5	1608.3	1612.5	1596.4	15.1	0.94

Figure 7-9 show the chromatograms obtained for Coca-Cola samples regular, light and zero, respectively. As can be seen in Fig. 7 regular coke has no ACS-K peak since it has sugar instead of LNCS, that fact could explain the slight variation at baseline. CAF is present qualitatively at the same level for regular, diet and zero Coca-Cola samples. Fig. 8 and Fig. 9 show ACS-K peaks at the same retention time and equal height, which correlates to the data in Table 14 that indicates similar concentration values for Zero Coke (142.64 mg/L) and Diet Coke (144.89 mg/L), contrary to the general belief both samples have the same concentration levels.

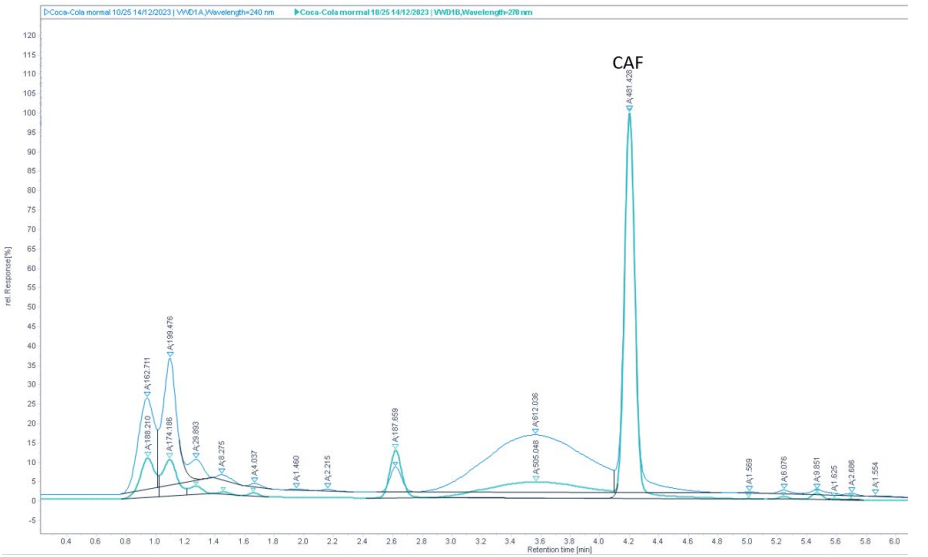


Figure 7. Representative chromatogram of sweeteners and additives from regular Coca-Cola sample.

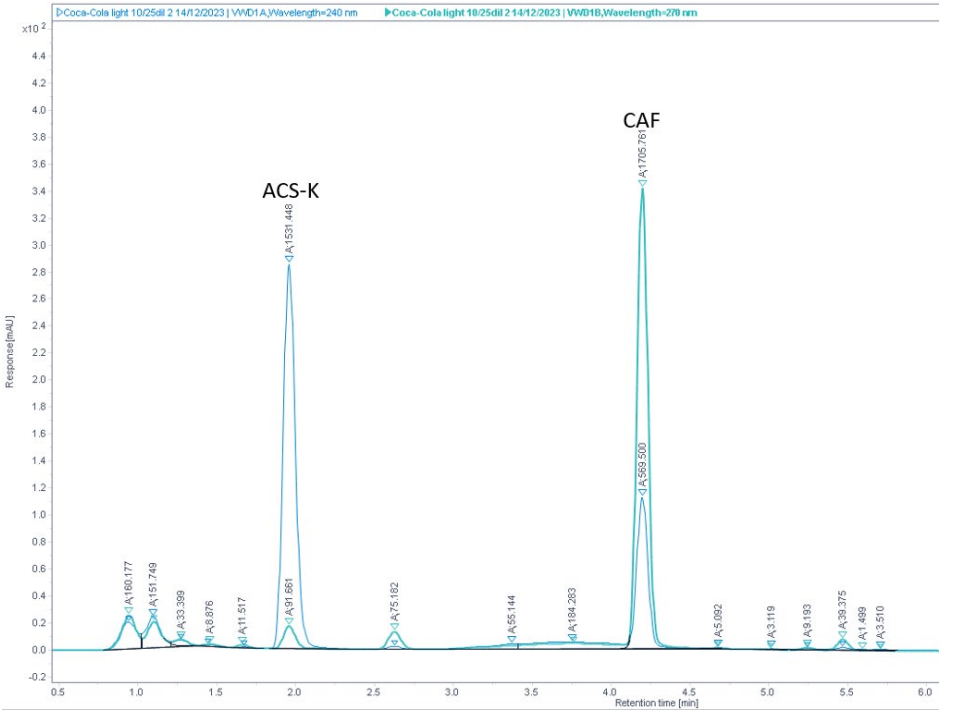


Figure 8. Representative chromatogram of sweeteners and additives from Coca-Cola Light sample.

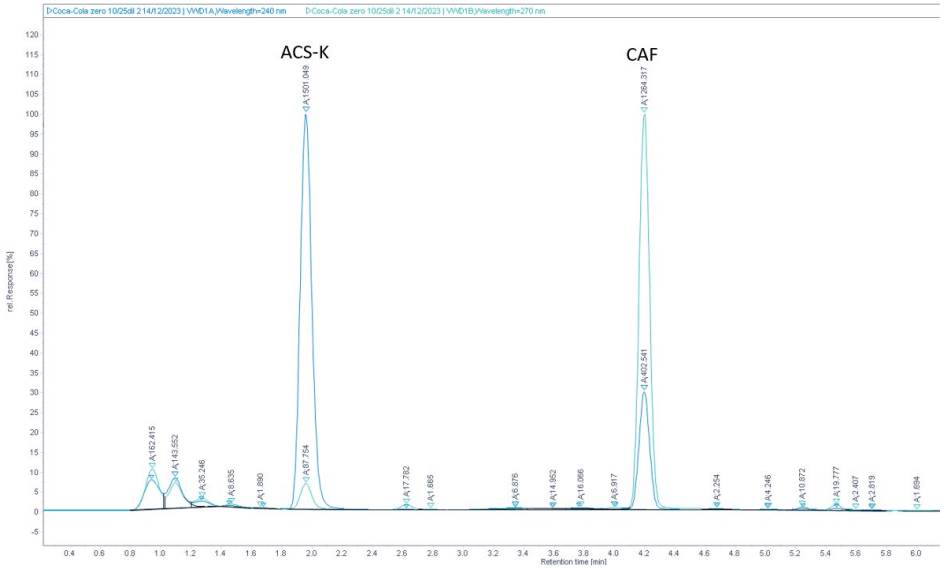


Figure 9. Representative chromatogram of sweeteners and additives from Coca-Cola Zero sample.

Pepsi (Fig. 10) shows similar level of CAF than the regular Coca-Cola sample. As both are cola soft drinks their matrix effect and additives concentrations should theoretically resemble as can be seen in Fig 7 and Fig 10. Nevertheless, ACS-K is present in lower levels compared to diet and sugar free Coca-Cola samples (88.2 mg/L). This beverage is arguably sweeter than its competitor since the presence of sweeteners enhance the sweetness strength of the preexistent sugars in the regular drink, regular Coca-Cola have no added sweeteners.

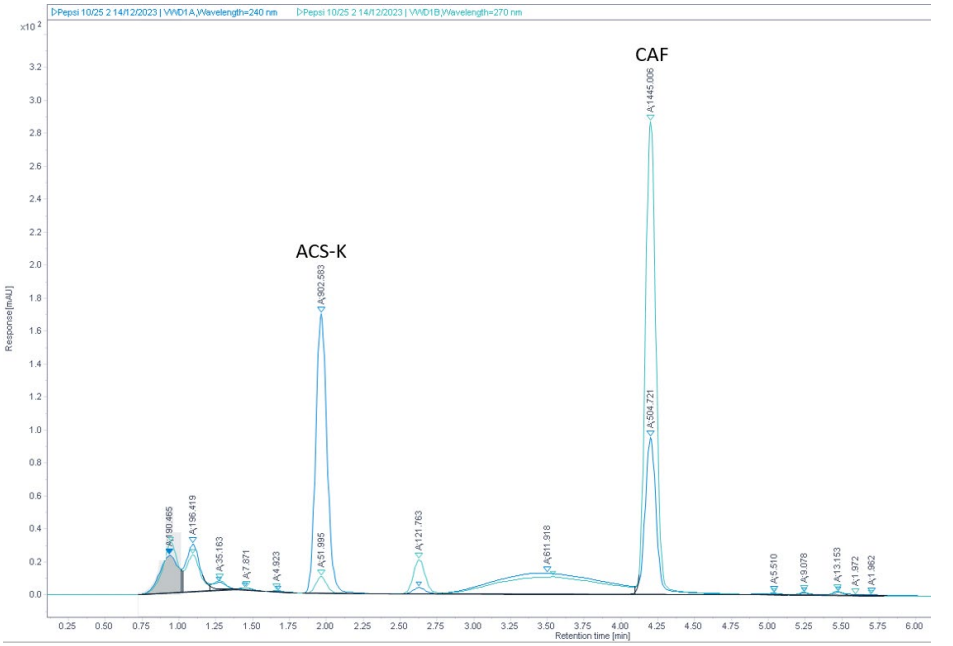


Figure 10. Representative chromatogram of sweeteners and additives from Pepsi sample.

Schweppes tonic shows a smaller peak for ACS-K (see Fig. 11) that correlates to a concentration of 115.7mg/L of ACS-K. The tonic contains quinine in great abundance that could be quantified in the future with the gradient proposed (see Table 4).

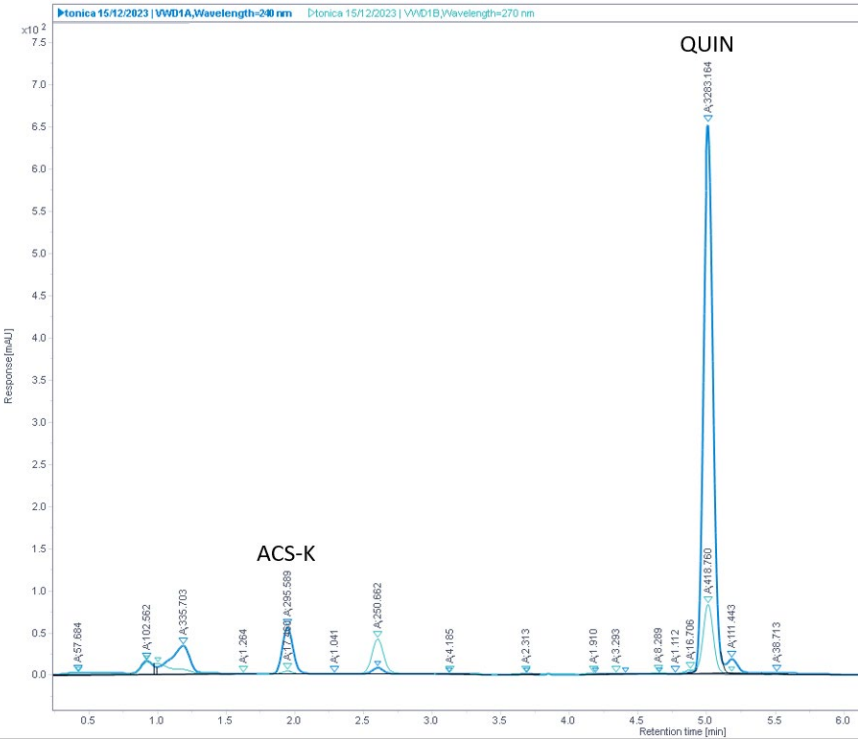


Figure 11. Representative chromatogram of sweeteners and additives from Schweppes tonic sample.

Orange and lemon Fanta samples have the same label specifications except for the type of citric juice added, so theoretically it should have equivalent matrix effects. As can be seen in Fig. 12 and Fig. 13 both samples have similar ACS-K concentration levels and rather high concentration levels of NHDC: 225.6 and 1689.8 mg/L for the orange drink, and 215.0 and 1596.4 mg/L for the lemon drink respectively. Lastly, it is noticeable that the height of ASC peak differs between each other which means that lemon flavor contains higher values of this compound than the orange flavor because its peak area is smaller.

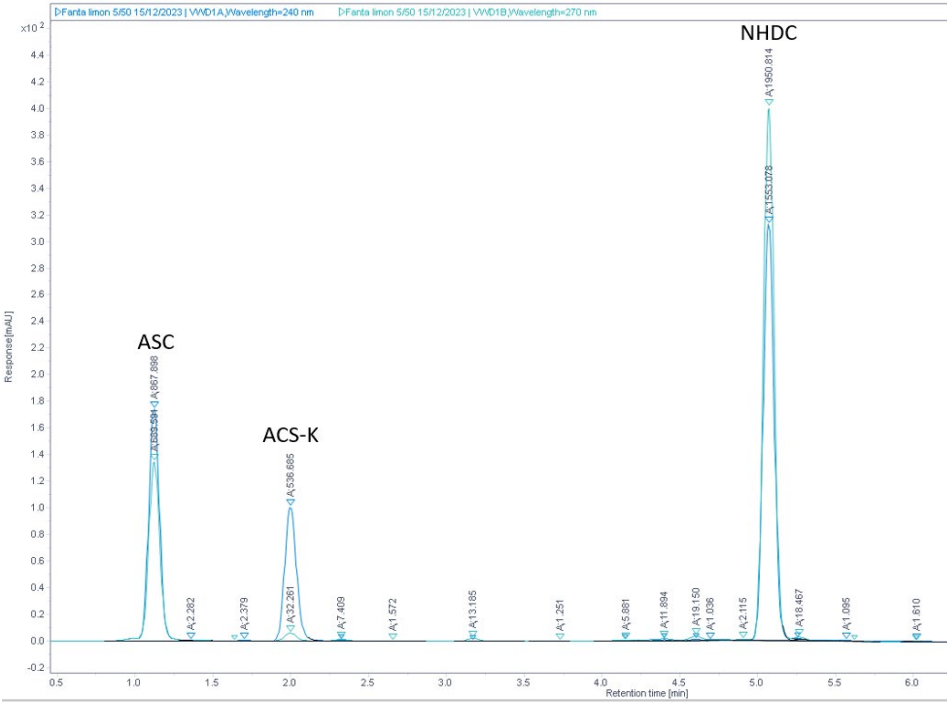


Figure 12. Representative chromatogram of sweeteners and additives from lemon flavour Fanta sample.

All the injections of the different samples were achieved with low variability for ACS-K as their RSD were below 1.11% except for Pepsi that was higher 3.41%. For NHDC, similar lower values of RSD were achieved (lower Than 0.94%). Retention time was also great in terms of variability, all the analyses showed comparable retention times with the standard solution as the variability between injections and samples for ACS-K only differ by 0.110 minutes and 0.003 minutes for NHDC.

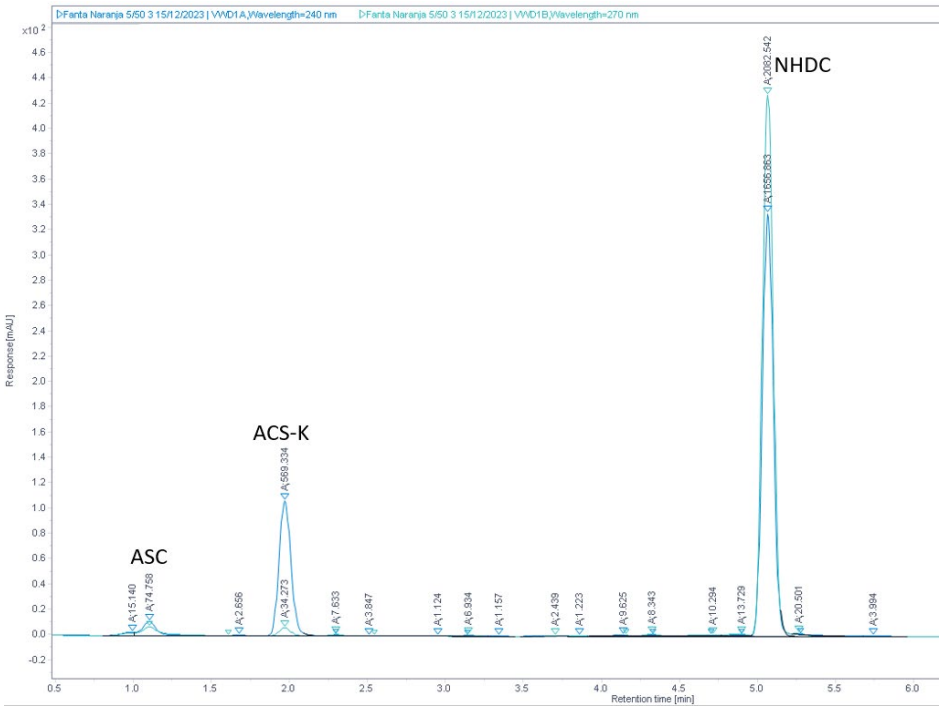


Figure 13. Representative chromatogram of sweeteners and additives from orange flavour Fanta sample.

The results found by this methodology indicate that all ACS-K concentration levels comply with the European legislation of the EFSA see Table 1, ACS-K must be below 350 mg/L. However, the concentrations of NHDC found in the Fanta samples outstrip by far the maximum level permitted by the EFSA (NHDC concentrations should not be greater than 30 mg/L). After the reliability of the quantitative determination was checked by comparison with results found in other published methods and their references [41] [42] [43] [44] [45], it is safe to say that determination of NHDC was not reliable, possibly, the sample treatment was still insufficient to eliminate some interferences of the matrix (is the most complex sample matrix analyzed) and they might coelute with NHDC enhancing its signal response, hence the area peak is much greater than it should be and in consequence, the concentration calculated is huge in comparison from the references. An exhaustive sample treatment by SPE should eliminate the possible interferences.

9. PRACTICE GUIDE FOR LQA LABORATORY

The research and the experimental procedure for the development of a RP-UHPLC/UV-vis method in gradient elution mode capable of separating up to three artificial sweeteners of a given sample for its multi-determination along with some other preservative and antioxidants has led to the creation of a novel practice guide suitable for the students of "Laboratori de Química Analítica, LQA" which is included in Annex I. Its objective is to enable the learning of how an HPLC-UV/vis system works through simple and relatively unsophisticated samples and sample treatments, in which the student will be able to grasp how routine analysis in soft drinks samples are performed in the industry.

10. CONCLUSIONS

A new RP-UHPLC/UV-vis method for the determination of sweeteners (ACS-K, SAC and NHDC) and various common additives (caffeine, quinine, benzoic acid and ascorbic acid) has been proposed.

With regards to the mobile phase compositions, an aqueous phosphate buffer of 0.1M at pH 4.00 as Solvent A and methanol as Solvent B have been suggested for the optimal separation of analytes. Methanol has been selected in front of acetonitrile to reduce the cost of analysis for its implementation in the LQA laboratory.

It has been necessary to operate in gradient elution mode due to great differences in the hydrophobicity of the compounds. The optimized gradient elution program allows the retention of the more polar analytes separating them from the elution front, while the high elution strength used at the end of the chromatogram allows the elution of the more hydrophobic compounds. The linear gradient permits the separation of the analytes with good resolution and short analysis time, less than 7 minutes.

Under the optimized conditions for the analysis of sweeteners, quinine and benzoic acid coelute. If both were present in the same sample and should be determined, a further optimization to improve their separation would be necessary.

Regarding the detection:

The two wavelengths selected (240 and 270 nm) have been chosen to allow the optimal detection of all the compounds. The selection has been based on the maximum intensity of the sweeteners and the best wavelength selectivity.

The proposed RP-UHPLC/UV-vis method has shown good sensitivity and reproducible results for ACS-K and NHDC.

LODs and LOQs obtained are relatively low (below 1.14 and 3.81 mg/L, respectively), which are enough for the detection of the sweeteners in soft drinks. The quantitation has been possible since the method shows good linearity within the working range studied with great repeatability of slope RSD (0.0098% for ACS-K and 0.58% for NHDC). Regarding the quantitation of sweeteners, the method is repeatable (intra-day RSD: for ACS-K <1.88%; NHDC <5.39%) and reproducibility (inter-day RSD: for ACS-K <7.40%; NHDC <2.7%).

Concerning the RP-UHPLC/UV-vis analysis of soft drink samples, the method enables the determination of the concentration levels in the samples which were in the range 88.2 - 225.7 mg/L for ACS-K, and 1596.4 - 1689.9 mg/L for NHDC. All the samples were below the EU maximum permitted levels for ACS-K, but the NHDC concentrations were greatly above the legislated levels. In this case, the result can indicate that there can be a problem with the sample treatment since Fanta samples are more complex and would require a more exhaustive treatment by SPE to eliminate the possible interferences in pursuance of reliable results.

Sweeteners like CYC and SCL that lack chromophore groups can be detected by UV-vis if derivatized to introduce a suitable chromophore in their structure. From literature, it seems that a relatively easy derivatization can be achieved by reacting CYC and SCL with NBD-F or PNBCl, respectively. Regarding ASP, even though it has a chromophore group, the wavelength range for maximum absorption is at 210 nm, which is too low for a direct analysis due to the high background signal. The derivatization of ASP can be done by reacting its hydrolysis products with NDA in the presence of cyanide ion in a mildly alkaline medium. Thus, derivatization will improve selectivity and sensitivity as an increase of wavelength causes considerable improvement of the baseline and conceivably mitigates the effect of the methanol gradient. These procedures are suggested as potential reactions for further studies and to implement new lab practices.

Lastly, it has been possible to write a practice guide based on the recent gradient HPLC-UV/vis method for the determination of sweeteners in soft drinks which is included in Appendix 1 for future undergraduate students of “Laboratori de Química Analítica, LQA”.

11. REFERENCES AND NOTES

[1] Roy. Moxham, “The great hedge of India,” p. 234, 2002, Accessed: Jan. 04, 2024. [Online]. Available: https://books.google.com/books/about/The_Great_Hedge_of_India.html?hl=es&id=sVWItAEACAAJ

[2] “The Cambridge World History of Food - Sugar.” Accessed: Jan. 04, 2024. [Online]. Available: https://web.archive.org/web/20120123183317/http://www.cambridge.org/us/books/kiple/sugar.htm

[3] A. C. Sylvetsky and K. I. Rother, “Trends in the consumption of low-calorie sweeteners,” Physiol Behav, vol. 164, no. Pt B, pp. 446–450, Oct. 2016, doi: 10.1016/J.PHYSBEH.2016.03.030.

[4] C. Pérez-Rodrigo et al., “Clustering of Dietary Patterns, Lifestyles, and Overweight among Spanish Children and Adolescents in the ANIBES Study,” Nutrients, vol. 8, no. 1, p. 11, Dec. 2016, doi: 10.3390/NU8010011.

[5] M. Nissensohn et al., “Beverage Consumption Habits and Association with Total Water and Energy Intakes in the Spanish Population: Findings of the ANIBES Study,” Nutrients, vol. 8, no. 4, Apr. 2016, doi: 10.3390/NU8040232.

[6] C. Piernas, S. W. Ng, and B. Popkin, “Trends in purchases and intake of foods and beverages containing caloric and low-calorie sweeteners over the last decade in the United States,” Pediatr Obes, vol. 8, no. 4, pp. 294–306, 2013, doi: 10.1111/J.2047-6310.2013.00153.X.

[7] F. Bellisle and A. Drewnowski, “Intense sweeteners, energy intake and the control of body weight,” Eur J Clin Nutr, vol. 61, no. 6, pp. 691–700, Jun. 2007, doi: 10.1038/SJ.EJCN.1602649.

[8] S. E. Swithers, “Artificial sweeteners produce the counterintuitive effect of inducing metabolic derangements,” Trends Endocrinol Metab, vol. 24, no. 9, pp. 431–441, Sep. 2013, doi: 10.1016/J.TEM.2013.05.005.

[9] A. D. Nichol, M. J. Holle, and R. An, “Glycemic impact of non-nutritive sweeteners: a systematic review and meta-analysis of randomized controlled trials,” Eur J Clin Nutr, vol. 72, no. 6, pp. 796–804, Jun. 2018, doi: 10.1038/S41430-018-0170-6.

[10] M. de L. Samaniego-Vaesken et al., “Added Sugars and Low- and No-Calorie Sweeteners in a Representative Sample of Food Products Consumed by the Spanish ANIBES Study Population,” Nutrients 2018, Vol. 10, Page 1265, vol. 10, no. 9, p. 1265, Sep. 2018, doi: 10.3390/NU10091265.

[11] “1. Food Additives and why they are Used,” Essential Guide to Food Additives, pp. 1–21, Feb. 2008, doi: 10.1039/9781847559234-00001.

[12] L. F. Capitán-Vallvey, M. C. Valencia, E. Arana Nicolás, and J. F. García-Jiménez, “Resolution of an intense sweetener mixture by use of a flow injection sensor with on-line solid-phase extraction Application to saccharin and aspartame in sweets and drinks,” Anal Bioanal Chem, vol. 385, no. 2, pp. 385–391, May 2006, doi: 10.1007/S00216-006-0346-0/FIGURES/4.

[13] L. Zhao and B. J. Tepper, “Perception and acceptance of selected high-intensity sweeteners and blends in model soft drinks by propylthiouracil (PROP) non-tasters and super-tasters,” Food Qual Prefer, vol. 18, no. 3, pp. 531–540, Apr. 2007, doi: 10.1016/J.FOODQUAL.2006.07.004.

[14] A. Zygler, A. Wasik, and J. Namieśnik, “Analytical methodologies for determination of artificial sweeteners in foodstuffs,” TrAC Trends in Analytical Chemistry, vol. 28, no. 9, pp. 1082–1102, Oct. 2009, doi: 10.1016/J.TRAC.2009.06.008.

[15] A. Wasik, J. McCourt, and M. Buchgraber, “Simultaneous determination of nine intense sweeteners in foodstuffs by high performance liquid chromatography and evaporative light scattering detection—Development and single-laboratory validation,” J Chromatogr A, vol. 1157, no. 1–2, pp. 187–196, Jul. 2007, doi: 10.1016/J.CHROMA.2007.04.068.
[16] D. J. Yang and B. Chen, “Simultaneous Determination of Nonnutritive Sweeteners in Foods by HPLC/ESI-MS,” J Agric Food Chem, vol. 57, no. 8, pp. 3022–3027, Apr. 2009, doi: 10.1021/JF803988U.
[17] N. Dossi, R. Toniolo, S. Susmel, A. Pizzariello, and G. Bontempelli, “Simultaneous RP-LC determination of additives in soft drinks,” Chromatographia, vol. 63, no. 11–12, pp. 557–562, Jun. 2006, doi: 10.1365/S10337-006-0793-Y/METRICS.
[18] A. Hannisdal, “Analysis of acesulfame-K, saccharin and preservatives in beverages and jams by HPLC.,” Z Lebensm Unters Forsch, vol. 194, no. 6, pp. 517–519, Jun. 1992, doi: 10.1007/BF01185474.
[19] E. Çubuk Demiralay, G. Özkan, and Z. Guzel-Seydim, “Isocratic separation of some food additives by reversed phase liquid chromatography,” Chromatographia, vol. 63, no. 1–2, pp. 91–96, Jan. 2006, doi: 10.1365/S10337-005-0683-8/METRICS.
[20] A. M. Di Pietra, V. Cavrini, D. Bonazzi, and L. Benfenati, “HPLC analysis of aspartame and saccharin in pharmaceutical and dietary formulations,” Chromatographia, vol. 30, no. 3–4, pp. 215–219, Aug. 1990, doi: 10.1007/BF02274550/METRICS.
[21] H. Matsumoto, K. Hirata, N. Sakamaki, K. Hagino, and H. Ushiyama, “[Simultaneous determination of neotame, alitame and aspartame in foods by HPLC].,” Shokuhin Eiseigaku Zasshi, vol. 49, no. 1, pp. 31–36, Feb. 2008, doi: 10.3358/SHOKUEISHI.49.31.
[22] Y. Zhu, Y. Guo, M. Ye, and F. S. James, “Separation and simultaneous determination of four artificial sweeteners in food and beverages by ion chromatography,” J Chromatogr A, vol. 1085, no. 1, pp. 143–146, Aug. 2005, doi: 10.1016/J.CHROMA.2004.12.042.
[23] M. Herrmannová, L. Křivánková, M. Bartoš, and K. Vytřas, “Direct simultaneous determination of eight sweeteners in foods by capillary isotachophoresis,” J Sep Sci, vol. 29, no. 8, pp. 1132–1137, May 2006, doi: 10.1002/JSSC.200500441.
[24] M. C. Yebra and P. Bermejo, “Indirect determination of cyclamate by an on-line continuous precipitation-dissolution flow system,” Talanta, vol. 45, no. 6, pp. 1115–1122, Apr. 1998, doi: 10.1016/S0039-9140(97)00219-1.
[25] J. L. Rufino, H. R. Pezza, and L. Pezza, “Flow-injection spectrophotometric determination of azithromycin in pharmaceutical formulations using p-chloranil in the presence of hydrogen peroxide,” Analytical Sciences, vol. 24, no. 7, pp. 871–876, Jul. 2008, doi: 10.2116/ANALSCI.24.871/METRICS.
[26] R. A. Medeiros, A. E. de Carvalho, R. C. Rocha-Filho, and O. Fatibello-Filho, “Simultaneous square-wave voltammetric determination of aspartame and cyclamate using a boron-doped diamond electrode,” Talanta, vol. 76, no. 3, pp. 685–689, Jul. 2008, doi: 10.1016/J.TALANTA.2008.04.015.
[27] M. Grembecka, P. Baran, A. Błaz ˙ewicz ·, Z. Fijałek, and P. Szefer, “Simultaneous determination of aspartame, acesulfame-K, saccharin, citric acid and sodium benzoate in various food products using HPLC-CAD-UV/DAD,” Eur Food Res Technol, vol. 238, pp. 357–365, 2014, doi: 10.1007/s00217-013-2111-x.
[28] S. Nojiri et al., “プレカラム誘導体化法を用いた HPLC による食品中のスクラロースの分析,” 食品衛生学雑誌, vol. 43, no. 5, pp. 289–294, Oct. 2002, doi: 10.3358/SHOKUEISHI.43.289.
[29] M. Lehr and W. Schmid, “Einfaches und spezifisches HPLC-Verfahren zur Bestimmung von Cyclamat in fruchtsafthaltigen Getränken nach Derivatisierung zu N,N-

Dichlorcyclohexylamin," Z Lebensm Unters Forsch, vol. 192, no. 4, pp. 335–338, Apr. 1991, doi: 10.1007/BF01202764.
[30] M. Hauck and H. Köbler, "Bestimmung von Cyclamat in komplexer Matrix mittels HPLC nach Vorsäulenderivatisierung mit 4-Fluoro-7-nitrobenzofurazon," Z Lebensm Unters Forsch, vol. 191, no. 4–5, pp. 322–324, Oct. 1990, doi: 10.1007/BF01202436.
[31] H. S. Lim, E. A. Choi, J. Y. Hwang, G. Lee, S. S. Yun, and M. K. Kim, "Improved method for the determination of 12 non-nutritive sweeteners and monitoring in various foods using liquid chromatography–tandem mass spectrometry," Food Additives & Contaminants: Part A, vol. 35, no. 9, pp. 1674–1688, Sep. 2018, doi: 10.1080/19440049.2018.1486043.
[32] A. Kirgöz et al., "A biosensor based on graphite epoxy composite electrode for aspartame and ethanol detection¨Ulkü detection¨ detection¨Ulkü," Anal Chim Acta, vol. 570, pp. 165–169, 2006, doi: 10.1016/j.aca.2006.04.010.
[33] H. Matsumoto, K. Hirata, N. Sakamaki, K. Hagino, and H. Ushiyama, "HPLC による食品中のネオテーム，アリテームおよびアスパルテームの同時分析法," 食品衛生学雑誌, vol. 49, no. 1, pp. 31–36, Feb. 2008, doi: 10.3358/SHOKUEISHI.49.31.
[34] J. McCourt, J. Stroka, and E. Anklam, "Experimental design-based development and single laboratory validation of a capillary zone electrophoresis method for the determination of the artificial sweetener sucralose in food matrices," Anal Bioanal Chem, vol. 382, no. 5, pp. 1269–1278, Jul. 2005, doi: 10.1007/S00216-005-3258-5/FIGURES/5.
[35] K. Hayakawa, T. Schilpp, K. Imai, T. Higuchi, and O. S. Wong, "Determination of Aspartic Acid, Phenylalanine, and Aspartylphenylalanine in Aspartame-Containing Samples Using a Precolumn Derivatization HPLC Method," J Agric Food Chem, vol. 38, no. 5, pp. 1256–1260, 1990, doi: 10.1021/JF00095A022/ASSET/JF00095A022.FP.PNG_V03.
[36] Y. Zhu, Y. Guo, M. Ye, and F. S. James, "Separation and simultaneous determination of four artificial sweeteners in food and beverages by ion chromatography," J Chromatogr A, vol. 1085, no. 1, pp. 143–146, Aug. 2005, doi: 10.1016/J.CHROMA.2004.12.042.
[37] Q. C. Chen and J. Wang, "Simultaneous determination of artificial sweeteners, preservatives, caffeine, theobromine and theophylline in food and pharmaceutical preparations by ion chromatography," J Chromatogr A, vol. 937, no. 1–2, pp. 57–64, Dec. 2001, doi: 10.1016/S0021-9673(01)01306-1.
[38] J. L. Rafferty, J. I. Siepmann, and M. R. Schure, "Mobile phase effects in reversed-phase liquid chromatography: A comparison of acetonitrile/water and methanol/water solvents as studied by molecular simulation," J Chromatogr A, vol. 1218, no. 16, pp. 2203–2213, Apr. 2011, doi: 10.1016/J.CHROMA.2011.02.012.
[39] M. Yang, S. Fazio, D. Munch, and P. Drumm, "Impact of methanol and acetonitrile on separations based on π–π interactions with a reversed-phase phenyl column," J Chromatogr A, vol. 1097, no. 1–2, pp. 124–129, Dec. 2005, doi: 10.1016/J.CHROMA.2005.08.028.
[40] X. Qiao, M. Ye, Y. H. Liang, W. Z. Yang, and D. A. Guo, "Retention behaviors of natural products in reversed-phase liquid chromatography using mobile phase comprising methanol, acetonitrile and water," J Sep Sci, vol. 34, no. 2, pp. 169–175, Jan. 2011, doi: 10.1002/JSSC.201000695.
[41] M. R. Castellar, J. L. Iborra, and I. Canales, "Analysis of Commercial Neohesperidin Dihydrochalcone by High Performance Liquid Chromatography," J Liq Chromatogr Relat Technol, vol. 20, no. 13, pp. 2063–2073, 1997, doi: 10.1080/10826079708005565.
[42] T. Pérez-Ruiz, C. Martínez-Lozano, V. Tomás, A. Sanz, and E. Bravo, "Quantitative assay for neohesperidin dihydrochalcone in foodstuffs by capillary electrophoresis," Chromatographia, vol. 51, no. 7–8, pp. 385–389, 2000, doi: 10.1007/BF02490473/METRICS.

[43] L. Chen, S. Zhu, C. Wang, and L. Chen, “Development of a HPLC-UV Method for the Separation and Quantification of Hesperidin, Neohesperidin, Neohesperidin Dihydrochalcone and Hesperetin,” Nat Prod Res, vol. 37, no. 10, pp. 1714–1718, 2023, doi: 10.1080/14786419.2022.2104275.

[44] R. Schwarzenbach, “Liquid chromatography of neohesperidin dihydrochalcone,” J Chromatogr A, vol. 129, no. C, pp. 31–39, Dec. 1976, doi: 10.1016/S0021-9673(00)87765-1.

[45] H. Montijano, F. Borrego, I. Canales, and F. A. Tomás-Barberán, “Validated high-performance liquid chromatographic method for quantitation of neohesperidine dihydrochalcone in foodstuffs,” J Chromatogr A, vol. 758, no. 1, pp. 163–166, Jan. 1997, doi: 10.1016/S0021-9673(96)00697-8.

12. ACRONYMS

ACS-K	Acesulfame-K
ACS	Ascorbic acid
ASP	Aspartame
BEN	Benzoic acid
CAF	Caffeine
CYC	Cyclamate
EFSA	European Food Safety Authority
FTIR	Fourier Transformed Infrared
HPIC-UV	High Pressure Ion Chromatography with ultraviolet detector
HPLC	High Performance Liquid Chromatography
LC-UV	Liquid chromatography with ultraviolet detector
LNCS	Low and No Calorie Sweeteners
NBD-F	4-fluoro-7-nitrobenzofuran
NDA	Naphthalene-2,3-dicarboxaldehyde
NHDC	Neohesperidin dihydrochalcone
RP-UHPLC	Reversed-Phase Ultra High-Performance Liquid Chromatography
PNBCl	p-nitrobenzyl chloride
SAC	Saccharin
SCL	Sucralose
SPE	Solid Phase Extraction
UE	European Union
UHPLC	Ultra High-Performance Liquid Chromatography
UV-vis	Ultraviolet-visible

APPENDICES

APPENDIX 1: PRACTICE GUIDE: DETERMINACIÓ D'ACESULFAME-K, CAFEÏNA I ÀCID ASCÒRBIC EN REFRESCOS.

En aquesta pràctica es realitza la determinació del contingut d'acesulfame-k (ACS-K), neohesperidina dihidrocalcona (NHDC), cafeïna i àcid ascòrbic en varies mostres representatives del sector dels refrescos. Coca-cola Light. Coca-Cola Zero, Pepsi, Fanta de taronja, Fanta de llimona i tònica Schweppes.

Instruments i condicions operatòries

- Cromatògraf de líquids Agilent 1260 Infinity II System, amb detector UV (UV-vis Agilent amb Sistema de detecció d'ultraviolat-visible de longitud d'ona variable).

- Columna: InfinityLab Poroshell 120 EC-C18 (100 mm longitud x 4.6 mm diàmetre intern x 2.7 µm de grandària de partícula superficialment porosa) d'Agilent.

- Cabal de la fase mòbil: 1 mL/min.

- Solvent A: NaH_2PO_4 0.1M dissolt en aigua qualitat i ajustat a pH 4, el pH amb HCl.

- Solvent B: Metanol qualitat HPLC.

- Mode d'elució: Gradient:

Taula 1. Gradient

Time (min)	A (%)	B (%)
0.00	85.0	15.0
1.00	85.0	15.0
4.00	20.0	80.0
7.00	20.0	80.0
15.00	85.0	15.0
16.00	85.0	15.0

- Volum d'injecció: 10 µL.

- Longitud d'ona de detecció: 240 nm i 270 nm.

Reactius

- Metanol de qualitat HPLC.
- Aigua de qualitat HPLC.
- Acesulfame-K de Supelco Analytical, USA.
- Cafeïna 98-101% de Panreac, Espanya.
- L-(+)-Àcid ascòrbic 99-100.5% d'Analar Normapur, Bègica.
- Neohesperidina Dihidrocalcona ≥ 95% de Sigma-Aldrich, USA.

Procediment

Es filtra la dissolució aquosa i el metanol, emprant un filtre de membrana de Niló de 0,45 µm. Per tal d'acondicionar la columna cromatogràfica, abans d'injectar qualsevol solució, cal fer circular la fase mòbil (85% de solvent A i 15% de solvent B) pel sistema cromatogràfic durant uns 15 minuts.

Es preparen les solucions patons per a l'anàlisi qualitativa i/o quantitativa o les mostres corresponents amb aigua qualitat HPLC. Prèviament a la seva injecció totes les dissolucions s'han de filtrar amb un filtre de xeringa amb una membrana de Niló de 0,45 µm.

Anàlisi qualitativa

Es preparen solucions patrons de 100 mg/L de cada un dels compostos i s'injecten en el cromatògraf per obtenir el temps de retenció de cadascun dels analits. A continuació s'injecta una mescla que conté tots els compostos a analitzar i s'identifica l'ordre d'elució

S'injecta un dels patrons d'una concentració elevada (> 10 mg/L) per mesurar els temps de retenció dels analits. L'ordre d'elució dels compostos és: àcid ascòrbic, acesulfame-K i cafeïna.

Anàlisi quantitativa

Es preparen mescles de dissolucions patró que continguin els analits a quantificar. L'interval de treball és entre 0.76-100 mg/L per la cafeïna, 2.28-100 mg/L per l'acesulfame-K i 4.10-100 mg/L per l'àcid ascòrbic. S'injecten les mostres per triplicat i els patrons de la recta de calibratge per tal de determinar la concentració dels compostos en base a l'àrea dels pics cromatogràfics obtinguts.

Tractament de mostra

Les mostres seleccionades es desgasen durant 15 minuts en un bany d'ultrasons i es fan les següents dilucions:

- Per la Coca-Cola Light, Coca-Cola Zero i Pepsi s'ha de fer una dilució 10/25.
- Per les Fantes es necessari diluir la mostra 5/50.
- La tònica Schweppes es pot injectar directament.

Prèviament a la seva injecció les dissolucions de les mostres s'han de filtrar amb un filtre de xeringa amb una membrana de Niló de de 0,45 µm