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Characterization and Green Extraction of Plant Bioactives: Encapsulation of Roselle Extract for Functional Dairy Dessert Development

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CONTENTS

1. Introduction and Objectives 3
2. Review of Literature 6
2.1 Polyphenols 6
2.2 Polyphenols of interest in this study 10
2.2.1 Chlorogenic acids 10
2.2.2 Anthocyanins 13
2.3 Caffeine—alkaloid rich in coffee matrices 16
2.4 Extraction of polyphenols from plant materials 17
2.5 Application of polyphenol-rich plant materials in dairy products 20
2.5.1 Interactions of dairy matrix components and polyphenols 23
2.5.2 Sensory acceptability of polyphenol-enriched dairy products…. 27
2.5.3 Challenges associated with polyphenol-enriched dairy product manufacturing 29
2.5.4 Túró Rudi 35
2.6 Encapsulation of plant extracts rich in polyphenols 35
3. Materials and methods 41
3.1 Materials and instruments 41
3.1.1 Chemicals 41
3.1.2 Plant materials 42
3.1.3 Food ingredients 42
3.1.4 Equipments 43
3.2 Sample preparation and extraction procedures 44
3.2.1 Preparation of roasted coffee extracts (conventional solvents)... 44
3.2.2 Preparation of ethanolic extracts of roselle calyx 45
3.2.3 Preparation of natural deep eutectic solvent extracts of green coffee beans 48
3.2.4 Preparation of natural deep eutectic solvent extracts of roselle calyx 49
3.3 Developing an HPLC method to analyze 5-CQA concentration in plant extracts 50
3.3.1 Preparation of 5-CQA standard 50
3.3.2 HPLC analysis of 5-CQA in plant extracts 51
3.4 Developing an HPLC method to analyze caffeine concentration in green and roasted coffee extracts 52

3.4.1 Preparation of caffeine standard 52
3.4.2 HPLC analysis of caffeine in green and roasted coffee extracts.... 52
3.5 Spectrophotometric analysis of phenolic compounds in plant materials extracted with natural deep eutectic solvents 53
3.5.1 Total phenolics in green coffee beans and roselle calyx 53
3.5.2 Determination of total flavonoid content in roselle calyx 54
3.5.3 Determination of total monomeric anthocyanin content in roselle calyx 54
3.5.4 Determination of DPPH radical-scavenging activity of roselle calyx 55
3.6 Encapsulation of the natural deep eutectic solvent extract of the roselle calyx 56
3.6.1 Determination of encapsulation efficiency 57
3.6.2 Drying yield of encapsulated powder 58
3.6.3 Moisture content of encapsulated powder 58
3.6.4 Determination of functional properties of encapsulated powder..... 59
3.6.5 Determination of powder properties and solubility 59
3.6.6 Determination of color stability (by spectrophotometric absorbance) at varying pH levels 60
3.7 Developing a dairy dessert (Túró Rudi) incorporating freeze-dried encapsulated powder of roselle 60
3.7.1 Preparation of enriched Túró Rudi 60
3.7.2 Analysis of antioxidant properties of Túró Rudi fortified with roselle encapsulated powder 61
3.7.3 Analysis of nutritional and sensory properties of Túró Rudi..... 62
3.8 Statistical Analysis..... 63
4. Results and Discussion..... 64
4.1 Quantification of 5-CQA and caffeine in roasted coffee extracts 64
4.2 Determination of 5-CQA concentration in ethanolic extracts of roselle calyx 69
4.2.1 HPLC conditions for the separation of 5-CQA 69
4.2.2 Optimization of the extraction conditions and quantification of 5-CQA in ethanolic extracts of roselle calyx 72
4.3 Analysis of phenolics and caffeine concentration in natural deep eutectic solvent extracts of green coffee beans..... 81
4.3.1 Total phenolics in green coffee bean extracts..... 81

4.3.2 Chlorogenic acid concentration in green coffee bean extracts.... 84
4.3.3 Caffeine concentration in green coffee bean extracts.... 85
4.3.4 Effect of extraction temperature on total phenolics, chlorogenic acid and caffeine concentrations of green coffee bean extracts.... 87
4.4 Phenolic compounds in natural deep eutectic solvent extracts of roselle calyx 89
4.4.1 Total phenolic content in roselle calyx extracts 89
4.4.2 Total flavonoid content in roselle calyx extracts 91
4.4.3 Total monomeric anthocyanin content in roselle calyx extracts.....93
4.4.4 Antioxidant activity of roselle calyx extracts 95
4.4.5 Chlorogenic acid content in roselle calyx extracts 97
4.5 Evaluation of encapsulated natural deep eutectic solvent extracts of roselle calyx 102
4.5.1 Encapsulation efficiency, drying yield, moisture content, visual characteristics and functional properties of encapsulated powder…..... 103
4.5.2 Powder properties and pH stability of encapsulated powder..... 110
4.6 Characteristics of Túró Rudi with roselle encapsulated powder.... 114
4.6.1 Functional properties of Túró Rudi with roselle encapsulated powder 114
4.6.2 Nutritional properties of Túró Rudi with roselle encapsulated powder 118
4.6.3 Sensory properties of Túró Rudi with roselle encapsulated powder 121
5. Conclusions 124
6. Detailed summary 126
7. New scientific results 129
8. Publications 130
8.1 Journal articles (Peer-reviewed indexed journals)..... 130
8.2 Journal articles (Peer-reviewed non-indexed journals) 131
8.3 Conferences attended 132
9. References 133
10. ACKNOWLEDGEMENT 172
11. APPENDICES 173
11.1 Sensory ballot sheet 173

ABBREVIATIONS

3-CQA	3-O-caffeoylquinic acid
4-CQA	4-O-caffeoylquinic acid
5-CQA	5-O-caffeoylquinic acid
CGA	Chlorogenic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSE	Delphinidine-3-sambuboide equivalents
ENC	Encapsulation
GA	Gum arabic
GAE	Gallic acid equivalents
Glu-CA	Glucose-citric acid
Glu-Glyc	Glucose-glycerin
Glu-LA	Glucose-lactic acid
HPH	High-pressure homogenization
HPLC	High-performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MD	Maltodextrin
NADES	Natural deep eutectic solvent
PDA	Photo-diode-array
RP	Reverse-phase
SD	Standard deviation
SE	Standard error
TFC	Total flavonoid content
TMA	Total monomeric anthocyanin
TPC	Total phenolic Content

Characterization and Green Extraction of Plant Bioactives: Encapsulation of Roselle Extract for Functional Dairy Dessert Development

ABSTRACT

This study explores the development of a green, efficient encapsulation (ENC) method for polyphenols extracted from roselle calyx, specifically targeting their application in functional dairy products. Key objectives comprised optimizing high-performance liquid chromatography-photo diode array (HPLC-PDA) methods for phenolic analysis, optimizing extraction and ENC parameters, and evaluating the functional, nutritional, and sensory properties of the final food product (Túró Rudi), added with encapsulated phenolic-rich roselle calyx extract. Initially, this study optimized an HPLC-PDA method for the analysis of chlorogenic acids (CGA), particularly its major isomer 5-o-caffeoylquinic acid (5-CQA), in conventional solvent extracts of roselle (*Hibiscus sabdariffa* L.) calyx and coffee (*Coffea arabica*) (roasted beans). Roasted coffee showed 3.7 to 6.3 mg/g 5-CQA content depending on the coffee type and extraction method, while 1.43 to 1.48 mg/g of 5-CQA was extracted from dried hibiscus flowers.

Secondly, this study investigated the potential of using natural deep eutectic solvents (NADESs) for the extraction of polyphenols from roselle calyx and green coffee beans. Results revealed that glucose–glycerin (Glu-Glyc) NADES is a promising, eco-friendly solvent for extracting phenolic compounds from green coffee beans, providing an alternative to 50% ethanol. Among the various NADES formulations (Glu-Glyc, glucose-citric acid—Glu-CA, glucose-lactic acid—Glu-LA), tested for the extraction of phenolics from roselle calyx, Glu-LA mixture demonstrated

superior extraction performance, achieving the highest total phenolic content (TPC) of 22.96±1.06 mg gallic acid equivalents—GAE/g at 50˚C, and notable total monomeric anthocyanin (TMA) (8.13±0.43 mg Delphinidine-3-sambuboide equivalents—DSE/g), total flavonoid content (TFC) (2.83±0.15 mg QE/g), and antioxidant activity (13.90±0.80 mg ascorbic acid equivalents—AAE/g). NADES also remained competitive with reference ethanolic solvents in terms of CGA extraction and provided comparable yields (2.50±0.02 mg CGA/g).

ENC of Glu-LA extracts of roselle calyx were assessed with maltodextrin (MD) and gum arabic (GA) in five ratios; (1:0, 1:3, 1:1, 3:1, and 0:1.). The 3:1 MD-to-GA blend offered the best ENC efficiency (99%), a good drying yield (85%), low moisture content (5.31%), excellent flow properties (Carr index 10%, Hausner ratio 1.11), and quick solubility (51 s). Despite diluting the phenolic content (2.50±0.08 mg GAE/g), ENC provided stable, free-flowing powders suitable for food and nutraceutical applications with modest antioxidant capacity (0.36±0.04 mg AAE/g). The encapsulated anthocyanins retained their color and stability best under acidic conditions (pH 1–2).

Incorporation of the encapsulated powder into Túró Rudi at 3% to 9% fortification led to increased protein content (from 14.66% to 15.60%), reduced fat (from 6.38% to 5.15%), and enhanced antioxidant activity (up to 1.31±0.01 mg AAE/g). Sensory analysis showed improved color and maintained acceptability, even at higher levels of fortification. The ENC matrix effectively protected bioactives during processing, ensuring their delivery in the final product. Overall, this research validates the use of NADES-based extraction and freeze-drying ENC as sustainable and efficient approaches for stabilizing polyphenols in functional foods. The

integrated method brought about an innovative, phenolic-enriched dairy dessert with enhanced nutritional, functional, and sensory attributes. Future work should focus on scaling up processes associated with assessing health benefits in human studies.

1. Introduction and Objectives

Interest in polyphenols for functional food applications is increasing due to their health benefits and industrial advantages. Chlorogenic acids (CGAs), especially 5-caffeoylquinic acid (5-CQA), and anthocyanins, offer antioxidant, antimicrobial, and anti-inflammatory effects, contributing to disease prevention (Pop et al., 2023; Lomovskiy et al., 2020; Rojas-González et al., 2022). Their incorporation into dairy products can enhance nutritional value and support chronic disease management, making diets healthier and more sustainable (Helal et al., 2022; Huang et al., 2023; Zhang et al., 2024). Plant-derived bioactives, unlike some synthetic drugs, tend to have fewer side effects (Budryn et al., 2013). Bioavailability is key to polyphenol benefits, but compounds like CGA and anthocyanins have poor solubility and instability, limiting their direct use in foods (Ianni et al., 2022). They may interact with food matrices or degrade during digestion, reducing effectiveness (Liu et al., 2016; Alongi et al., 2019; Oliveira et al., 2019). Accurate quantification of phenolic compounds is essential for food applications. High-performance liquid chromatography (HPLC) is the standard method due to its simplicity, speed, and cost-effectiveness (Yang et al., 2026).

Encapsulation (ENC) in food-grade materials improves stability and delivery. Common methods include spray drying, freeze-drying, coacervation, and ionic gelation; newer options like electrospinning, electrospraying, and $nano_{ENC}$ target better distribution and organ-specific

delivery (Tylkowski & Tsibranska, 2016; Szpicer et al., 2025). More research is needed to optimize delivery systems for CGA and anthocyanins. Selecting an appropriate solvent is crucial before ENC to maximize phenolic yield and activity. Ethanol and aqueous ethanol are common, food-grade choices (Tripathi et al., 2025). Traditional solvents like acetone and methanol are effective but pose toxicity and environmental issues, prompting the use of natural deep eutectic solvents (NADESs), which are non-toxic, biodegradable, and effective at dissolving various phenolic compounds (Xue et al., 2022; Okeke et al., 2025). NADESs can be tailored for specific targets and, despite high viscosity, can be optimized with water (Hijo et al., 2022). Being food-grade, NADESs allow extracts to be used directly in food or pharmaceutical applications without complex removal processes.

This study selected coffee (green and roasted beans) and roselle calyxes for polyphenol extraction due to their high CGA content. Several solvents (methanol, water, aqueous ethanol, and NADES) were tested for the extraction of phenolics. Quantification of CGA in coffee and roselle extracts was performed using HPLC with photodiode-array (PDA) detection, ensuring peak purity, enabling differentiation of CGA isomers, and allowing simultaneous caffeine analysis at 270 nm. Caffeine, another key bioactive, was measured in coffee extracts to assess extract quality, flavor, and health benefits. Spectrophotometric methods were used to determine phenolic content and antioxidant activity of NADES extracts from green coffee and roselle. The main aim was to develop an ENC method for polyphenol-rich roselle calyx extract and apply it to a dairy-based functional food. Addressing gaps in existing research on encapsulating roselle extracts with freeze-drying—which preserves heat-

sensitive phenolics—this study developed a freeze-drying method for roselle calyx phenolics extracted with glucose and lactic acid-based NADES.

The objectives of this study can be summarized as follows.

1. To develop and optimize an HPLC-PDA method for the analysis of CGA in roselle calyx and coffee extracts.
2. To optimize the ENC parameters for a NADES-based roselle calyx extract with phenolic compounds.
3. To develop a dairy-based functional food that contains encapsulated polyphenols.
4. To analyze the antioxidant properties of the developed functional food occupying 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging approach.

To achieve these objectives, the following tasks were accomplished.

- An HPLC-PDA method was optimized to analyze CGA concentration in ethanolic extracts of hibiscus calyx, methanolic and water extracts of roasted coffee, and NADES extracts of green coffee beans and roselle calyx.
- Three glucose-based NADESs—glucose-glycerin (Glu-Glyc), glucose-citric acid (Glu-CA), and glucose-lactic acid (Glu-LA)—were evaluated for their phenolic extraction efficiency from roselle calyx.
- A NADES (Glu-LA) extract of roselle calyx was encapsulated with a freeze-drying approach using maltodextrin (MD) and gum Arabic (GA) as excipients.

- Lyophilized roselle calyx extract was used to enrich a Hungarian dairy-based dessert (Túró Rudi), and its impact on functional, nutritional, and sensory properties was analyzed.

2. Review of Literature

2.1 Polyphenols

Interest in using polyphenols in functional food applications is increasing due to their potential health benefits and their industrial applications. Polyphenols are among the most important plant metabolites in both human and animal diets, as they exhibit a wide array of biological activities, including antioxidant, antimicrobial, and anti-inflammatory actions (Lomovskiy et al., 2020; Pop et al., 2023). Among these, antioxidant activity is the most attractive property of polyphenols. Their capacity to scavenge reactive oxygen species such as superoxide anions and hydroxyl radicals confers this characteristic, which helps prevent and treat conditions like cancer, diabetes, and cardiovascular disease that are driven by oxidative cell damage (Lomovskiy et al., 2020; Condezo-Hoyos et al., 2021; Lang et al., 2024). Polyphenols are abundant in fruits such as berries, watermelons, grapes, apples, and in vegetables such as broccoli, carrots, and onions, as well as in whole grains, nuts, and beverages such as red wine, coffee, and tea (Grosso, 2018; Lang et al., 2024). Polyphenols can be categorized into two major groups based on their molecular structure: Flavonoids and non-flavonoids (Pop et al., 2023) (Figure 1). Flavonoids can further be classified into six groups: flavanols, flavanones, flavones, isoflavones, flavonols and anthocyanins. Non-flavonoids can be classified into four groups: phenolic acids, coumarins, stilbenes, and lignans (Pop et al., 2023).

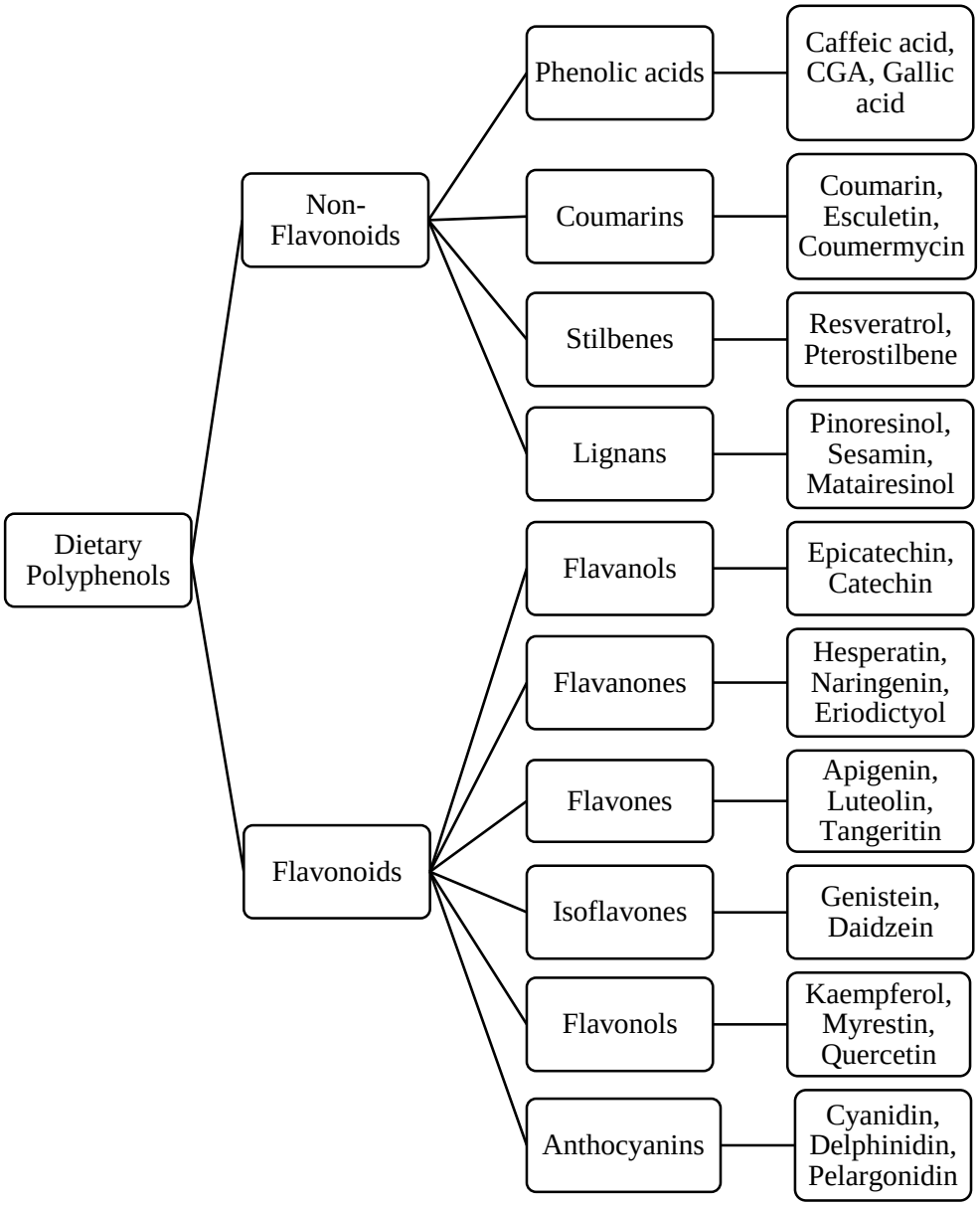


Figure 1 Classification of dietary polyphenols (Source: Pop et al., 2023)

Recent studies highlight the potential health benefits of green coffee beans, particularly in weight management, heart health, neurological conditions, and blood sugar regulation, attributed to its rich bioactive

compounds, including caffeine, diterpenes, and CGA. Green coffee bean extracts are commonly used as supplements for the prevention and treatment of non-communicable diseases such as type 2 diabetes, high blood pressure, and cardiovascular disease (Munyendo et al., 2021; Khalili-Moghadam et al., 2023). Caffeine, which is an alkaloid, constitutes approximately 1-2.5% of coffee's dry matter, while CGA, specifically caffeoylquinic acids like 3-CQA, 4-CQA, and 5-CQA, account for 4-12% of dry matter (Farah & Donangelo, 2006). Notably, 5-CQA, the primary phenolic compound, exhibits redox properties and accumulates in coffee beans as they mature, contributing to the extracts' health benefits by reducing lipid levels and improving insulin sensitivity (Acidri et al., 2020; Tripathi et al., 2025; Chandimala & Ajtony, 2025). *Coffea arabica* and *Coffea canephora* var. *robusta* are the two most widely consumed varieties of roasted coffee, with arabica known for its superior flavor, while robusta is popular for its stronger taste profile due to higher caffeine levels. Coffee's 5-CQA content affects its flavor profile, which varies with concentration and brewing time and can range from astringent to sweet, or sour (Izawa et al., 2010; Ludwig et al., 2014). Total CGA in regular coffee drinks ranges from 2 to 7 mg/g, while decaffeinated coffee drinks have slightly lower levels (0.8 to 6.2 mg/g) (Lu et al., 2020). Caffeine content can vary considerably between instant and freshly brewed coffee (Ludwig et al., 2014). However, the roasting process typically reduces phytochemical content by up to 66%, with 5-CQA being particularly affected (Acidri et al. 2020).

Roselle, a prominent species among over 300 hibiscus varieties that belong to the Malvaceae family prevalent globally, is particularly favored in tropical and subtropical regions for its edible flower, generally utilized

in herbal tea preparations due to its therapeutic properties (Riaz & Chopra, 2018). Roselle's calyx is a key ingredient in herbal teas. Likewise, it is used as a coloring and flavoring agent in food, medicine, and cosmetics, levered for its appealing flavor and distinctive reddish-purple hue (Alañón et al., 2020; Chongwilaikasem et al., 2024). Roselle calyces are rich in bioactive compounds, including flavonoids (e.g., quercetin, catechin, and anthocyanins), as well as phenolic acids, which contribute to their antioxidant, anti-inflammatory, antihypertensive, and hypoglycemic effects (Duque-Soto et al., 2023; Banwo et al., 2022). Amongst these phenolics, anthocyanins are the predominant (>99%) based on HPLC analysis (Wang et al., 2024b). The main anthocyanins in hibiscus calyx are delphinidin-3-sambubioside and cyanidin-3-sambubioside, along with their derivatives, which contribute to the calyx's distinctive red color and biological activities (Cira-Chávez et al., 2025). Literature indicates that *H. sabdariffa* L. calyx contains delphinidin-3-sambubioside and cyanidin-3-sambubioside in an 80:20 ratio (Cira-Chávez et al., 2025). Recent studies highlight the antibacterial activity and moderate DPPH radical-scavenging ability of hibiscus extracts (Chongwilaikasem et al., 2024), further reinforcing its potential in functional foods and cosmetics (Paraı´so et al., 2019; Wairata et al., 2022). The extraction methods for obtaining these bioactive compounds significantly impact yield and efficacy, with traditional water extraction techniques yielding less compared to organic solvent methods, which have been more extensively explored (Wairata et al., 2022). Researchers have documented the potential of organic solvents such as ethanol, methanol, hexane, and chloroform in extracting phenolics from plant extracts. Particularly, polar solvents like ethanol and methanol have been effective in extracting hydrophilic compounds, while non-polar

solvents tend to extract more lipophilic substances (Kebede et al., 2025; Shraim et al., 2025). For example, Banwo et al. (2022) extracted 7.25 mg GAE/g of phenolics from roselle using 80% (v/v) methanol.

2.2 Polyphenols of interest in this study

2.2.1 Chlorogenic acids

CGA is a family of quinic acid esters with substituted cinnamic acids that are created by substituting the aromatic ring and contain isomers and epimers in the cyclohexane portion (Mok et al., 2022; Sakai et al., 2022). Caffeoylquinic acid, dicaffeoylquinic acid, and feruloylquinic acid are the three primary classes of CGA. Neochlorogenic acid—3-CQA, cryptochlorogenic acid—4-CQA, and CGA—5-CQA are the three most prevalent CQA isomers found in plant sources (Sakai et al., 2022). In reverse phase (RP)-HPLC, CGA isomers are separated based on varying elution orders influenced by conditions such as polarity. In RP-HPLC, analytes with higher polarity tend to distribute more into the eluent, eluting faster from the column. Among the isomers, 5-CQA and 4-CQA, which possess one axial and one equatorial hydroxyl group, are less hydrophilic than 3-CQA with two equatorial hydroxyls. Residual silanol group activity also affects elution order, with findings indicating it to be 3-CQA>5-CQA>4-CQA under specific conditions (Deineka et al., 2019). Additionally, the pH of the mobile phase significantly affects the retention of these acidic analytes (Jeon et al., 2019). The most abundant CGA isomer is 5-CQA (Figure 2), a major polyphenol found in many plants such as green coffee beans, hibiscus, and honeysuckle, with a significant dietary intake closely linked to coffee consumption (Aree, 2023; Grujic et al., 2015). It is extensively studied due to its notable health benefits. For example, 5-CQA has attracted scientific interest mainly because of its

antioxidant, anti-inflammatory, antibacterial, anti-cancer, and analgesic activities, as well as its positive regulatory effects on gut microflora.

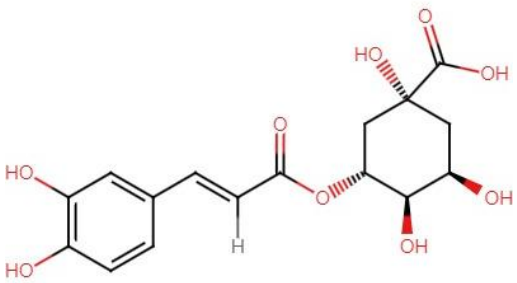


Figure 2 Molecular structure of CGA (5-O-caffeoylquinic acid). Source: (Chandimala & Ajtony, 2025)

CGA exhibits multiple biological activities, primarily due to its antioxidant and anti-inflammatory properties. Its antioxidant action is attributed to its catechol structure, which enables free radical scavenging, reduction of reactive oxygen species, and metal ion chelation (Rojas-González et al., 2022). Recent studies have shown that CGA decreases expression of the Keap1 and nuclear factor erythroid 2-related factor 2 signaling pathways, thereby reducing malondialdehyde levels by upregulating the mRNA expression of a set of endogenous antioxidant enzymes, including catalase, superoxide dismutase, glutathione, and glutathione peroxidase, which are key agents in the detoxification of polyaromatic hydrocarbons (Wang et al., 2022; Xu et al., 2025). Moreover, CGA has demonstrated protective effects against various oxidative stressors, including tert-butylhydroperoxide, hydrogen peroxide, and ferrous sulfate. CGA's chelation of transition metals such as Fe^{2+} disrupts oxidative chain reactions and inhibits low-density lipoprotein oxidation (Wang et al., 2022). CGA also exerts anti-inflammatory effects by

suppressing pro-inflammatory cytokines, inhibiting lipopolysaccharide-induced cyclooxygenase activation, and elevating reduced glutathione levels. According to the study by Huang et al. (2023), CGA suppresses lipopolysaccharide-induced cyclooxygenase-2 expression by attenuating the nuclear factor-κB and c-Jun N-terminal kinase/activator protein-1 signaling pathways. Similarly, CGA has been shown to reduce malondialdehyde levels in wound tissues and enhance wound-healing effects (Rojas-González et al., 2022; Huang et al., 2023). Furthermore, CGA has been shown to exhibit antitumor activity through DNA damage mitigation, modulation of apoptotic pathways, and inhibition of carcinogen-induced molecular events (Rojas-González et al., 2022). CGA's intestinal barrier function and positive impact on gut microbiota composition make it a promising natural remedy for conditions such as inflammatory bowel disease and colorectal cancer (Rojas-González et al., 2022; Xu et al., 2025). CGA has shown therapeutic properties against type-2 diabetes by regulating glucose homeostasis. For example, Zuñiga et al. (2018) reported that administering CGA to patients with impaired glucose tolerance resulted in decreased fasting plasma glucose levels (5.7 to 5.5 mmol/L), insulinogenic index (0.71 to 0.63), and improved insulin sensitivity/Matsuda index (1.98 to 2.30) in anthropometric evaluations. This observation can be attributed to CGA's ability to regulate carbohydrate and lipid metabolism by increasing the mRNA expression of key mediators of lipid metabolism, including peroxisome proliferator-activated receptor-α and liver X receptor-α, inhibiting the activity of the glucose-6-phosphatase enzyme to reduce hepatic glucose output, and decreasing the expression of sodium-dependent glucose transporters in the small intestine to reduce glucose absorption (Zuñiga et al., 2018). Based

on many *in vitro* and *in vivo* studies, CGA's ability to improve endothelial function and reduce insulin resistance are critical mechanisms that enhance cardiovascular protection (Rojas-González et al., 2022). The neuroprotective functions of CGA have led to its use in treating cerebrovascular diseases and improving cognitive function (Johal et al., 2025). The literature reveals that CGA can reduce oxidative damage in the cerebellum caused by methotrexate, a drug with harmful side effects used in treating cancers and rheumatoid arthritis. For instance, Lee et al. (2012) found that CGA inhibits matrix metalloproteinases and reduces brain damage, blood-brain barrier damage, and brain edema. In addition, CGA demonstrates neuroprotective effects against pro-inflammatory cytokine-induced neurodegenerative diseases (Lee et al., 2012). While these findings underscore CGA's broad therapeutic potential, translating them into dairy applications will depend on preserving these functions within food matrices and validating them in human studies. Therefore, regular consumption of a diet rich in 5-CQA is known to reduce the risk of many non-communicable diseases. For therapeutic purposes, daily intake recommendations range from 13.5 mg to 1200 mg (Budryn et al., 2013). Existing research indicates that CGA is a phenolic compound with a well-established safety profile that does not exhibit toxicity or negative effects in healthy human cells and tissues (Budryn et al., 2013). A workable method for providing health benefits without raising safety concerns is to incorporate CGA-rich natural ingredients into food products such as dairy at dosages corresponding to the suggested daily intake.

2.2.2 Anthocyanins

Anthocyanins are a type of flavonoid, a class of phenolic compounds. Over 600 anthocyanins are found in various plant sources. The chemical

structure of anthocyanin is made up of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts, along with a sugar moiety (Mazza & Miniati, 1993). Anthocyanidin is the aglycone or sugar-free form of anthocyanin—glycoside. The main groups of anthocyanidins include cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Khoo et al., 2017; Ma et al., 2025). As anthocyanidins are sugar-free aglycone equivalents of anthocyanins, they become more hydrophobic due to the absence of highly water-interactive –OH groups (Khoo et al., 2017). However, due to the unique chemical structure of anthocyanidin core with both weak acidic and basic nature, they are generally poorly soluble in pure water, but readily soluble in polar organic solvents such as ethanol, methanol or acidified (<pH 3.0) water (Khoo et al., 2017; Qi et al., 2022).These plant pigments give fruits, flowers, and leaves their red, purple, blue, and black colors. They are most concentrated in berries, hibiscus calyx, and red cabbage. In some plant sources, for instance, purple sweet potatoes, di- or tri-glycosylated acyl forms derived from the main groups of anthocyanins are found, which differ from those in berries (Xu et al., 2015). Anthocyanins' color changes with pH: in acidic environments, they turn red, while in alkaline conditions, they appear blue or purple (Khoo et al., 2017).

Anthocyanins have diverse biological functions, such as antioxidant, anti-inflammatory, and anti-tumor effects. Research shows their role in extending lifespan and in preventing or treating aging-related diseases, including cardiovascular, neurodegenerative, metabolic, skeletal diseases, cancer, and eye disorders (Khoo et al., 2017; Ma et al., 2025). Anthocyanins can enhance antioxidant responses by promoting nuclear translocation of nuclear factor erythroid 2-related factor 2 and activating

enzymes like superoxide dismutase and glutathione peroxidase. They also chelate metal ions, thereby reducing the production of free radicals (Casedas et al., 2018). The neuroprotective effects of anthocyanins stem from their rapid absorption and ability to cross the blood-brain barrier, allowing them to act as direct scavengers of reactive oxygen species. *In vitro* studies show that anthocyanins prevent intracellular calcium overload, thereby mitigating excitotoxicity and neurodegenerative diseases (Salehi et al., 2020). Moreover, activation of nuclear factor erythroid 2-related factor 2 inhibits the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein-3 inflammasome, reducing apoptosis and inflammatory responses by modulating caspase-1 and cytokine signaling (Cui et al., 2018). Chronic inflammation is known to cause many chronic illnesses such as diabetes, cardiovascular disease, and cancer. Anthocyanins suppress the activation of the transcription factor nuclear factor-κB, which regulates genes involved in the inflammatory response, such as inducible nitric oxide synthase, cyclooxygenase-2, and various proinflammatory cytokines (e.g., tumour necrosis factor-α, interleukin-1β, and interleukin-6). They inhibit certain mitogen-activated protein kinase signaling cascades, thereby reducing the expression of proinflammatory cytokines, and cyclooxygenase-2. Additionally, anthocyanins directly inhibit cyclooxygenase-1 and cyclooxygenase-2, resulting in reduced-prostaglandin-E2 production (Hassimotto et al., 2013). They also suppress nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein-3 inflammasomes by activating nuclear factor erythroid 2-related factor 2 and the thioredoxin-1/thioredoxin-interacting protein inhibitory complex, thereby plays a crucial role in inflammatory

apoptosis by facilitating the release of cytokines such as interleukin-1β, interleukin-18, and caspase-1, and has been linked to various diseases (Cui et al., 2018). In cancer prevention, anthocyanins primarily act through pathways regulating cell survival, proliferation, apoptosis, and inflammation, with particular emphasis on nuclear factor-κB and cyclooxygenase-2 signaling (Salehi et al., 2020). To exploit the documented health benefits of anthocyanins, the recommended dietary intake is 50 mg/day. As there are no reported adverse health effects, anthocyanins do not have a tolerable maximum intake limit (Xue et al., 2022). Incorporating anthocyanin-rich plant extracts into dairy products is a possible approach to enhance the usability of functional properties of anthocyanins, and thus, the interactions between dairy matrix components and anthocyanins and their alterations with processing conditions need to be further investigated.

2.3 Caffeine—alkaloid rich in coffee matrices

Caffeine is a naturally occurring purine alkaloid that serves as the most widely consumed central nervous system stimulant across the globe, primarily sourced from coffee beans, tea leaves, and cocoa pods (Evans et al., 2024; Tawfeeq et al., 2025). While historically viewed with skepticism regarding chronic health outcomes, a substantial body of epidemiological and clinical literature now supports its therapeutic value when consumed in moderate quantities (Poole et al., 2017). Caffeine's framework consists of a heterocyclic purine core—specifically a fused pyrimidinedione and imidazole ring system—substituted with three hydrophobic methyl groups (Evans et al., 2024; Tawfeeq et al., 2025). In its pure form, caffeine exists as an odorless, white crystalline powder characterized by a distinctly bitter taste profile (Tawfeeq et al., 2025).

Due to its moderately lipophilic nature, caffeine is rapidly absorbed through the gastrointestinal tract, reaching peak plasma concentration within 30 to 60 minutes and readily crossing the blood-brain barrier without cellular resistance (Nehlig, 2018). The primary physiological effects of caffeine are driven by its role as a competitive adenosine receptor antagonist (Evans et al., 2024). By structurally mimicking adenosine, caffeine prevents the binding of this somnogenic neurotransmitter, thereby neutralizing its downstream inhibitory signals on the central nervous system (Nehlig, 2018). This antagonistic action indirectly facilitates a surge in the extracellular concentrations of key excitatory neurotransmitters, including dopamine, norepinephrine, and serotonin (Jeon et al., 2019; Evans et al., 2024). Systematic reviews confirm that acute doses improve working memory, shorten choice reaction time, and sustain attentional throughput during periods of sleep deprivation (Evans et al., 2024; Poole et al., 2017). At much higher concentrations, caffeine acts as a phosphodiesterase inhibitor, increasing intracellular cyclic adenosine monophosphate levels, and promotes intracellular calcium mobilization (Nehlig, 2018). Epidemiological cohorts suggest a robust, dose-dependent inverse correlation between habitual caffeine intake and the incidence of neurodegenerative disorders (Nehlig, 2018). In addition, large-scale prospective studies establish that moderate daily intake (3–5 cups of coffee) yields a J-shaped protective effect against cardiovascular diseases and stroke (Poole et al., 2017).

2.4 Extraction of polyphenols from plant materials

Solvent extraction is a prominent method for isolating phytochemicals with antioxidant properties from plants, with the solubility of phenolic compounds influenced by their chemical structure and modifications such

as esterification or glycosylation (Taheri & Jafari, 2019). Various factors like temperature, extraction time, pH, and solvent properties also affect phenolic solubility and bioactive compound extraction (Furia et al., 2021; Chandimala et al., 2025). Phenolic compounds are generally considered hydrophilic. Overall, solvents with medium polarity, such as ethanol, methanol, and acetone, mixed with water, have been recognized to give higher yields of total phenolics and antioxidant activity in plant extracts (Tripathi et al., 2025). For example, Banwo et al. (2022) extracted 7.25 mg of gallic acid equivalents (GAE)/g of phenolics from roselle using 80% (v/v) methanol. Research indicates that polar solvents, including methanol, effectively extract hydrophilic compounds, while non-polar solvents like ethyl acetate are suited for lipophilic compounds (Kebede et al., 2025). To improve the extraction yield, various novel techniques have been investigated in recent studies. For example, Silva et al. (2023) revealed that 0.4 mg/g of CGA can be extracted from apple pomace using ultrasonically assisted extraction, which is significantly higher compared to pressurized liquid extraction (0.29 mg/g), pressurized liquid extraction combined with solid phase extraction (0.31 mg/g), and magnetic stirrer extraction (0.3 mg/g). Mok et al. (2022) extracted higher CGA levels (0.48 mg/g) from dried buds of *Hibiscus syriacus* using subcritical water at 190˚C for 10 min. Such methods often yield concentrated extracts compared to conventional extraction methods (e.g., Soxhlet extraction and heat reflux extraction), which are time-consuming and require large volumes of organic solvents (Meng et al., 2023). However, despite the effectiveness of organic solvents, their toxicity poses safety concerns in food applications, in addition to their volatility, flammability, and low degradability (Kebede et al., 2025; Shraim et al., 2025); hence, their

potential toxicities in food applications must be considered important safety concerns.

Recent advancements in green chemistry have led to the development of NADES as safer alternatives to conventional organic solvents (Xue et al., 2022). Composed of non-toxic, biodegradable, and easily available ingredients like sugars, salts, and amino acids, NADES are recognized for their low freezing points, cost-effectiveness, and environmental safety (Loukri et al., 2022). Interestingly, different hydrogen bond donors (e.g., organic acids, sugars) and hydrogen bond acceptors (e.g., choline chloride) can be mixed at various molar ratios to synthesize NADES with varying capacities to extract phenolic compounds from plants (Wang et al., 2024a). For example, Koh et al. (2024) summarized the applications of various sugar-based NADES used for extracting phenolics from different plant materials. Studies indicate that sugar-based NADES, such as those made from glucose and fructose, demonstrate high efficiency in extracting bioactive compounds, particularly phenolics and flavonoids, while maintaining low toxicity and costs, positioning them as effective green solvents for phytochemical applications. The ability of NADES to dissolve a wide range of biomolecules with excellent solubility and thermal stability, while remaining low in toxicity and cost, makes them a notable green solvent for use in phytochemical extraction from various plant materials (Okeke et al., 2025). In a study, Meng et al. (2023) extracted CGA from *Eucommia ulmoides* leaves using NADES with various compositions of hydrogen bond donors and hydrogen bond acceptors, employing ultrasonic wave-enhanced adsorption and desorption techniques with macroporous resins. Using a NADES composed of choline chloride and malic acid in a 1:1 ratio resulted in an extraction yield

of 42 mg/g of CGA from *E. ulmoides* leaves. When the extract was applied in milk-based systems, it improved the antioxidant capacity of the product without compromising sensory quality. Miao et al. (2024) developed a water-in-oil high internal phase emulsion with exceptional thermal, centrifugal, freeze-thaw, and storage stability, using glucose and sucrose-based NADES to load high levels of curcumin at an ENC efficiency (85-99%). In a study, Kurtulbaş et al. (2022) isolated 31.8 mg GAE of total phenolics and 2.9 mg of cyanidine-3-glucoside equivalents from 1 g of dry roselle calyx using a citric acid and ethylene glycol-based NADES with microwave-assisted extraction. Similarly, Alañón et al. (2020) demonstrated that 24.4 mg/g of phenolic acids and 6.0 mg/g of flavonoids can be extracted from roselle calyx using an oxalic acid and potassium chloride-based NADES with microwave-assisted extraction. When it comes to roselle, some limitations persist in extensive studies exploring different NADES mixtures for extracting phenolic compounds. For instance, choline chloride, the most studied ingredient in NADES mixtures used in recent plant extractions, has drawbacks such as high viscosity, potential harm when combined with certain ingredients, and thermal instability at higher temperatures, which restrict its industrial use (Marchel et al., 2022). Additionally, recent NADES-based studies on roselle (Alañón et al., 2020; Kurtulbaş et al., 2022) utilize microwave-assisted extraction, making them costly and challenging for large-scale industrial applications.

2.5 Application of polyphenol-rich plant materials in dairy products

Plant materials rich in polyphenols, particularly CGA and anthocyanins, such as green coffee, white radish, pomegranate peel, goji

berry, and *Cudrania tricuspidata* leaf, have been used in dairy formulations to enhance their antioxidant profile and health-promoting potential (Dönmez et al., 2017; Oh et al., 2016; Gamage et al., 2024). These ingredients are added in various forms—aqueous extracts, powders, or encapsulated formulations—and incorporated into yogurt, cheese, or dairy beverages before or after heat processing or fermentation (Oh et al., 2016; El-Sayed and El-Sayed, 2024; Gamage et al., 2024). In most cases, polyphenols are extracted from plant sources as the first step when applying them for food or pharmaceutical applications. Advanced extraction methods, such as microwave-assisted extraction, ultrasound-assisted extraction, and NADES, have been successfully employed to obtain high-purity polyphenols from various plant sources, including berry fruits, apple pomace, eucommia leaves, and hibiscus buds (Mok et al., 2022; Meng et al., 2023; Silva et al., 2023; Gamage et al., 2024). Çam et al. (2014) developed an ice cream incorporating spray-dried pomegranate peel, which showed improved antioxidant capacity and sensory acceptability. Recent studies reveal that free-form or encapsulated hibiscus extracts can be used in probiotic dairy matrices like yogurt to improve antioxidant, antimicrobial, and sensory properties (Shin et al., 2021; Stella et al., 2023). These applications show that polyphenol-rich plant extracts can provide functional benefits to dairy products, including enhanced oxidative stability, improved bioactivity, and potential health effects (Gamage et al., 2024; Yang et al., 2025). However, further research is needed to evaluate the impact of dairy processing conditions on polyphenol stability and to optimize delivery systems for industrial applications.

Recent studies have demonstrated the potential of fermented dairy matrices in supporting the functional activity of plant-derived bioactives. Fermentation creates a favorable environment for both CGA and anthocyanins, which are more stable under acidic conditions due to the low pH and complex matrix of yogurt, which protects phenolic compounds while milk proteins, large peptides, and fat globules help protect these compounds during digestion, enhancing the integrity of these molecules, thereby increasing their bioaccessibility (He et al., 2016; Helal et al., 2022). Lactic acid bacteria present in fermented products can activate phenolics via enzymatic transformation, converting glycosylated or bound forms into more bioavailable aglycones (Sukhikh et al., 2019). For instance, Yusufoğlu et al. (2025) revealed that fermentation, which involves lactic acid bacteria or yeast (*Saccharomyces cerevisiae*), can enhance the stability and bioavailability of anthocyanins through microbial metabolism by converting anthocyanin-3-O-glucoside into carboxy-pyranoanthocyanin derivatives, which unveil strong antioxidant and anti-inflammatory properties. For example, incorporating green coffee bean extract into fermented milk significantly enhanced its antioxidant capacity (Carmo et al., 2022). Similarly, incorporating 0.5% spray-dried green coffee bean extract (containing 0.46 g/g of CGA) improved the antioxidant capacity and sensory acceptability of yogurt. In a study, Pandey et al. (2021) developed fermented dairy products (yogurt and buttermilk) incorporating 7.5% black carrot concentrate with high consumer acceptability. Fortified dairy products had significantly higher polyphenol content, particularly 24.52–113.27 mg/100g of anthocyanins, and antioxidant activity. In a study, Kabakci et al. (2020) reported that fortification of kefir with 25% plant juices rich in anthocyanins (black

carrot, black mulberry, pomegranate and strawberry) caused an increase (1.8–4.8 times) in antioxidant activity. However, authors reported a reduction of TMA content in juices during storage due to the degradation of anthocyanins, copigmentation, or polymerization associated with the sugar content and microbial metabolites in kefir. Gamage et al. (2024) developed yogurt and fermented milk containing 131.6 mg of cyanidine-3-glucoside equivalents/kg of anthocyanins by incorporating 2 g/100 g of black goji berry. The authors revealed that at higher anthocyanin concentrations, they become well-stabilized through intramolecular interactions and intermolecular associations with other matrix components. These examples highlight the synergistic relationship between dairy matrices and plant bioactives.

2.5.1 Interactions of dairy matrix components and polyphenols

The stability and functionality of polyphenols in dairy systems are significantly influenced by their interaction with matrix components, particularly proteins and fats. Careful formulation of polyphenol-enriched dairy products is required not only because it greatly affects polyphenol stability and bioaccessibility, but also the dairy emulsion stability.

Non-covalent bonds such as hydrogen bonds and Van der Waals forces are predominant between CGA and α-casein and CGA and α-lactalbumin complexes, whereas hydrophobic interactions are predominant between CGA and β-casein and CGA and β-lactoglobulin (Liu et al., 2016). Additionally, covalent bonds form between CGA and milk proteins via nucleophilic amino acid residues (e.g., cysteine), which are only partially stable during enzymatic protein hydrolysis. Covalent interactions, which are irreversible, play an important role in changing the food structure (e.g., texture, rheological properties) (Poojari et al., 2023).

However, these mechanisms are influenced by factors such as pH, temperature, and the proteins involved (Carmo et al., 2022). Coffee-based dairy beverages (e.g., macchiato and latte brews) are produced by combining coffee with skim or whole milk, along with other additives such as creamers, sweeteners, and stabilizers (Dupas et al., 2006). To secure CGA and other coffee phenolics during digestion, the effect of creamers in coffee-milk mixtures has been studied recently. Yu et al. (2021) reported that CGA bioaccessibility improved from 27% (instant coffee alone) to 40–52% with creamers and up to 84% with skim milk. After digestion, ferric reducing antioxidant power decreased by 25% in instant coffee, while the decrease was lower in the other beverages with dairy ingredients (12-16%), suggesting that creamers retain antioxidants during digestion. In a study, Helal et al. (2022) found that yogurt fortified with coffee extract exhibited increased antioxidant capacity after digestion due to the release of CGA from milk protein complexes, as the previously bound CGA-protein complexes are detangled during digestion. Processing induces non-covalent interactions between CGA and milk proteins, safeguarding CGA from oxidative damage during digestion and heat treatments. These reversible interactions are largely disrupted during digestion by protease activity, releasing bioactive CGA (Dupas et al., 2006; Liu et al., 2016). Therefore, it is suggested that CGA-milk protein interactions improve the bioaccessibility of CGA after digestion, although they initially reduce the CGA content and its solubility, presenting a negative viewpoint (Tagliazucchi et al., 2012; Alongi et al., 2019). However, these phenomena remain controversial, highlighting the importance of further studies.

These polyphenol-protein interactions not only enhance the physicochemical stability of dairy emulsions but also the potential for

beneficial structural modifications in milk proteins. For example, irreversible covalent complexes formed between CGA and specific proteins, such as lactoferrin, have been shown to enhance protein retention under gastrointestinal conditions (Li et al., 2024). These effects offer promising avenues for enhancing both functional and technological attributes of CGA-fortified dairy products. Although milk fat's interaction with CGA is less explored, Alongi et al. (2019) found that increasing fat levels (up to 7.1%) in CGA-fortified dairy systems improved micellarization and CGA complexation with milk proteins, thus enhancing CGA stability. Tagliazucchi et al. (2012) found that CGA bioaccessibility increased with the fat content in milk, whereas no relationship was observed between CGA bioaccessibility and protein, carbohydrate, and calcium contents. This implies that the bioaccessibility of CGA in fermented dairy systems is improved by the protection provided by dairy matrix components, followed by concurrent release of CGA from the matrix for absorption.

Recent studies analyzing the effects of food matrix components, particularly glucose, fructose, starch, and different proteins, on anthocyanin stability after simulated digestion have shown that these components in foods protect against anthocyanin degradation during digestion (Sengul et al., 2014; Oliveira et al., 2019). Interactions between anthocyanins and dairy proteins are primarily non-covalent, reversible forces—hydrophobic interactions and hydrogen bonding—which form stable complexes that improve pigment stability, antioxidant capacity, and overall functionality. These bindings generally involve a protein's hydrophobic regions, which help protect anthocyanins from degradation, particularly in acidic, dairy-based food systems (He et al., 2016; Wang et

al., 2024b; Jing and Giusti, 2005; Van de Langerijt et al., 2023). This fact was supported by Zheng et al. (2009), who showed the positive influence of casein and whey proteins on the absorbance and antioxidant activity of bog bilberry anthocyanin extract. He et al. (2016) reported that more stable complexes are formed between casein and anthocyanins, which can reduce the anthocyanin degradation of grape skin extract at pH 6.3 induced by thermal treatment (80 ˚C × 2 h), oxidation (0.005% H_2O_2 × 1 h), and photo illumination (5000 Lux × 5 d). In a study that combined molecular docking and multiple spectral analyses, Wang et al. (2024b) revealed that both whey and casein proteins in milk enhance the stability of roselle anthocyanins during *in vitro* simulated digestion, encouraging roselle anthocyanin applications in dairy products. Although anthocyanin stability and functionality are preserved by the dairy matrix proteins, the effects or alterations on solubility, digestibility, and overall functionality of proteins should also be studied when anthocyanin-incorporated dairy products are developed. Yang et al. (2025) found that spray-drying-induced protein oxidation in milk powder can be reduced by incorporating anthocyanins, as these inhibit protein cross-linking and aggregation, thereby reducing protein oxidation products and increasing free sulfhydryl content. In addition, the fortification elevated specific amino acid levels (e.g., tyrosine, cysteine, methionine, aspartic acid). More interestingly, proteins with reduced oxidation promoted the growth of beneficial human gut bacteria, including *Bifidobacterium* and *Alistipes*, upon fermentation. The interactions between milk fat and anthocyanins have been studied only in limited studies, and, hence, remain controversial. For instance, Van de Langerijt et al. (2023) demonstrated that the addition of milk into freeze-dried blackberry blends resulted in increased bioaccessibility of

anthocyanins while lowering their bioavailability due to the diminished permeability through Caco-2 cells caused by milk fat interactions with anthocyanins. Fallah et al. (2020) also reported lower levels of malondialdehyde following dietary intake of anthocyanins, suggesting that anthocyanins aid in reducing lipid oxidation. These results suggest that anthocyanin incorporation improves the functionality of macromolecules in food products, particularly processed dairy products, and enhances the antioxidant activity of the final product.

2.5.2 Sensory acceptability of polyphenol-enriched dairy products

The inclusion of polyphenols such as CGA can affect the flavor, color, and aroma of dairy products. For instance, chlorogenic lactones and phenylindanes can impart a pronounced bitterness when roasted coffee is incorporated into dairy products. Lighter coffee roasts generally have lower bitterness than dark roasts, and they can be used in milk infusions to reduce bitterness. In addition, whole milk blends with higher fat content help mask the bitterness in roasted coffee (Wang et al., 2022). The color of CGA-rich plant extracts may also affect the appearance of lighter dairy products, making them slightly tinted/green-colored due to oxidative reactions between CGA and amino acids forming trihydroxy benzacridine derivatives (Liang & Were, 2018; Rahpeyma & Sekhavatizadeh, 2020). Recent studies indicate that a red pigment with minimal pH and temperature dependence can be produced by oxidative coupling of CGA and tryptophan. This can be applied at permitted doses as low as 0.01% to impart a natural red color to dairy products and is expected to become a novel natural pigment that can replace synthetic azo pigments, which have caused serious toxicity (Wang et al., 2022). Adding anthocyanins to dairy products can enhance their sensory acceptability, particularly by

improving color, as these natural pigments can impart an appealing red, purple, or pink hue (Tian et al., 2022; van de Langerijt et al., 2023). Nevertheless, their color stability is affected by pH; acidic products, such as yogurt, are more suitable for adding anthocyanin-rich plant extracts than neutral milk. Moreover, anthocyanin-rich plant extracts improve the sensory acceptability of various dairy products by masking off-flavors and conveying more appealing fruity or floral notes, especially at moderate concentrations (up to 7.5%) (Pandey et al., 2021). However, higher concentrations may convey an unappealing taste to the product. Besides, it is crucial to carefully formulate techniques, such as ENC, to control the exposure of these polyphenols to unfavorable extreme temperatures or light, which may diminish their functionality. Protein-polyphenol affinity, which is the tendency of polyphenols to interact with proteins, plays an important role in the textural attributes of functional dairy incorporated with plant ingredients. For instance, interactions between polyphenols and milk proteins can form complexes that may not fully dissolve in the product, leading to sedimentation and altering its viscosity and mouthfeel (e.g., enhancing the thickness of yogurt or affecting the creaminess of dairy beverages), resulting in a modified sensory experience. These interactions involve both covalent and non-covalent bonds, altering the solubility of milk proteins and leading to the formation of large insoluble structures (Carmo et al., 2022). For instance, according to Acharjee et al. (2021), fortifying yogurt with orange pomace powder caused a significant reduction in the firmness of the yogurt. On the contrary, Afshari and Fadaei (2021) reported an increase in dry matter content and viscosity in milk enriched with bitter orange peel extract, which affected the product's general acceptability. In a study, Pimpley et al. (2022) reported an

increased syneresis effect in yogurt supplemented with levels of green coffee bean extract above 2%. However, lower supplementation levels (0.5%) resulted in the most acceptable gel matrix and overall textural attributes, attributed to the protein-polyphenol affinity. In contrast, Dönmez et al. (2017) reported that syneresis in yogurt can be significantly decreased by incorporating green coffee powder and green tea powder at 2% and 0.02% rates, respectively.

2.5.3 Challenges associated with polyphenol-enriched dairy product manufacturing

The successful development of dairy products incorporating polyphenols depends on how they behave during processing and on the dairy matrix's influence on their bioaccessibility. Bioaccessibility—the ratio of phenolic concentration after intestinal digestion to the original—is commonly assessed using *in vitro* digestion models. Bioavailability is the fraction of digested phenolics that enter the systemic circulation (Teixeira et al., 2024).

CGA is known to be sensitive to oxidative degradation, isomerization, and transesterification under heat, oxygen, and light (Shi et al., 2007; Zhang et al., 2024). For instance, temperatures exceeding 121˚C can lead to the thermal degradation and isomerization of CGA. In oil-in-water dairy emulsions, high-pressure homogenization (HPH) is frequently employed to stabilize the system, although this process can facilitate interactions between phenolics and milk constituents, such as proteins and enzymes. In a study, Qie et al. (2022) studied the *in vitro* bioaccessibility of coffee polyphenols in heat-treated skim milk-based coffee beverages and reported that moderate heat (e.g., 25˚C for 30 min) preserved CGA more effectively than higher heat treatments (90˚C for 30 min and 121˚C for 15

min). Quan et al. (2020) found that the bioaccessibility of CGA in all coffee-milk blends was lower than that of coffee without milk (31.7 mg/L) due to the protein-polyphenol affinity, indicating a limitation in developing coffee-milk infusions. Coffee-milk blends processed with HPH and thermal treatment exhibited decreased absolute bioaccessibility of CGA when whole milk was used, whereas skim milk formulations better retained CGA (Quan et al., 2020). This can result from the lowered negative interference of fat and other fat-soluble compounds in skim milk with CGA compared to whole milk, under HPH and thermal processing conditions (Alongi et al., 2019). Further, Quan et al. (2020) highlight the importance of optimizing dairy formulation and adjusting the pH in heat-treated dairy beverages to preserve the health benefits of CGA. Additionally, pH adjustment can prevent clouding and curdling in CGA-enriched canned dairy beverages caused by increased acid levels during heat treatments. Maintaining an appropriate pH and carefully selecting the timing of CGA addition (e.g., before or after thermal treatment) can further minimize degradation. These strategies require optimization to reduce CGA loss through oxidation, lactonization, or decarboxylation during production. Liu et al. (2020) proposed protein–CGA–dextran conjugates as emulsion stabilizers, improving interfacial properties and antioxidant capacity. More particularly, recent research suggests that such conjugates can expose internal protein amino acid residues to a polar environment, reduce interfacial tension, and increase antioxidant capacity to varying degrees (Liu et al., 2020), demonstrating their potential for use in functional dairy products and warranting further exploration.

Duarte & Farah (2011) reported that consuming coffee with milk can impair the bioavailability of CGA in humans. Specifically, the amount of

CGA and CGA-related metabolites recovered in urine was lower (40%) in all subjects when coffee was consumed with milk than when coffee was consumed alone (68%). In an *in vitro* digestion model, the bioaccessible CGA content decreased from 80.2 to 53.0 and 69.5 μmol/200 mL in coffee without milk and coffee with whole milk infusion, respectively (Tagliazucchi et al., 2012).

In fermented dairy systems, fermentation with certain lactic acid bacterial strains may reduce CGA levels, as these microbes can metabolize CGA into caffeic and quinic acid via cinnamoyl esterase activity. For example, Oh et al. (2016) reported a significant decrease in the CGA levels in yogurt after fermenting milk supplemented with *Cudrania tricuspidata* leaf extract because *Lactobacillus gasseri* used as starter cultures metabolized CGA, while increasing the probiotic bacterial count in yogurt. More precisely, the proteolytic activity of *L. gasseri* was improved by *C. tricuspidata* supplementation, increasing the levels of peptides and amino acids in the developed yogurt, enhancing antioxidant activity, and positively contributing to the final product's functional profile. These findings support the notion that there is synergy between probiotic cultures and CGA-rich plant extracts when they are added simultaneously to fermented dairy products. However, milk proteins and microbial proteins can bind to CGA and precipitate, adversely affecting its recovery and bioactivity (Lu et al., 2020).

Similarly, anthocyanins are notably sensitive to thermal processing and extreme levels of pH, oxygen, light, metal ions, ascorbic acid, and sulfur dioxide, which can cause their chemical instability. For example, at visible wavelengths, the maximum absorbance of petanin and cyanidin 3-glucoside occurs at pH values below 4.0 and above 6.0, with a gradual

decline (Wu et al., 2015). Anthocyanins are generally unstable under weakly acidic or neutral pH conditions, and heat treatments above 80˚C (He et al., 2016). At higher temperatures, anthocyanin degradation occurs through deglycosylation, yielding anthocyanidin glycosides, which can further convert into various derivatives or transform into chalcones, and later into coumarin glycoside derivatives (Xue et al., 2024). The high instability of anthocyanins under processing conditions challenges their practicality as commercial ingredients in processed foods. Thermal degradation of anthocyanins in high-temperature processing dairy products follows first-order kinetics. However, their stability can be considerably improved through interactions with dairy proteins such as whey and casein, and occasionally by maintaining an acidic pH (He et al., 2016; Wang et al., 2024b; Yang et al., 2025). By storing anthocyanin-enriched heat-treated dairy products at 2–4˚C, the structural stability of anthocyanins can be further maintained (Xue et al., 2024). Various stabilization strategies, such as combining with other food additives, micro$_{ENC}$, structural modifications, and co-pigmentation, have shown potential to enhance anthocyanin stability (He et al., 2016). For instance, Wu et al. (2015) reported that yeast mannoproteins considerably enhance the thermal stability of anthocyanins by 4- to 5-fold while preserving their antioxidant properties, suggesting that yeast mannoproteins are potential ingredients to improve anthocyanin functionality. Recent studies indicate that adding β-cyclodextrin, alginate, or ferric ions can improve the thermal stability of anthocyanins in weakly acidic (pH>4) food systems that undergo mild heat treatments (e.g., 60˚C for 80 min) (Wu et al., 2015). However, there is a research gap regarding the protective role of these techniques on anthocyanins, which warrants further investigation. In

addition, anthocyanins can negatively affect the microorganisms in fermented dairy products due to their acidity and antimicrobial attributes. For example, Gamage et al. (2024) reported a significant decrease in viable cell counts during storage of fermented milk and yogurt containing black goji berry anthocyanin extract, although the final products contained an acceptable viable cell count of 7 log colony-forming units/g.

Studies suggest that CGA undergoes differential absorption kinetics, characterized by rapid gastric uptake in the stomach, where minor gastric absorption of intact CGA occurs, followed by a more prolonged, slower absorption phase within the small intestine, which serves as the primary site for active and passive absorption of CGA (Rojas-González et al., 2022). The remaining CGA that reaches the colon undergoes biotransformation by gut microbes into caffeic acid and quinic acid. Only one-third of the CGA absorbed from the intestine enters the bloodstream (Nallamuthu et al., 2015). The absorbed CGA can be completely utilized, hydrolyzed, and further interact with compounds such as sulfates or glucuronic acid, or oxidized or metabolized by the intestinal microbiota (Wang et al., 2022). A recent experiment on CGA bioavailability revealed that only 0.8% of CGA was recovered from urine, and 57% of total CGA intake was derived from microbial metabolites, indicating a link between the metabolic capacity of gut microflora and CGA bioavailability (Gonthier et al., 2003). When it comes to anthocyanins, the literature reports bioavailability ranging from 0-2%, indicating that only a minor fraction reaches target tissues, possibly due to high conjugation, microbial metabolism, and excretion via the excretory system (Oliveira et al., 2019; Teixeira et al., 2024). However, certain limitations in anthocyanin bioavailability studies exist; for instance, evaluations have focused only

on the native forms of anthocyanin and single-site absorption (Oliveira et al., 2019). Recent studies using simulated *in vitro* digestion models demonstrate that auto-association of anthocyanins with saliva enhances their stability, although their stability during later gastric digestion remains complex and depends on the anthocyanin source and complexity. Mice-based models suggest that initial absorption of anthocyanin (approximately 25%) occurs in the stomach (Salehi et al., 2020). In most cases, gastric-phase digestion did not affect anthocyanin stability (Zhao et al., 2017; Sirisena et al., 2018). Nevertheless, studies revealed that intestinal digestion significantly reduced the amount of anthocyanins, attributed to the presence of pancreatin and higher intestinal pH, which destabilized and made them more easily degradable (Sirisena et al., 2018; Oliveira et al., 2019). Approximately 15% of anthocyanins is absorbed in the small intestine. The high polar nature and low lipid solubility can be primary causes of the difficulty in the direct absorption of anthocyanins by human cells (Van de Langerijt et al., 2023). Studies demonstrate that anthocyanins in plasma are primarily present as their metabolites (e.g., methylated derivatives), which have lower biological activity than the original compounds (Salehi et al., 2020; Xue et al., 2022). However, recent studies suggest that the acylation pattern of some anthocyanins can enhance stability and biological activity, and this is still under investigation (Oliveira et al., 2019). Additionally, interactions between CGA or anthocyanins and milk proteins (α-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin) can affect these phenolics' bioaccessibility and antioxidant effectiveness (Dupas et al., 2006). Therefore, preserving polyphenols such as CGA and anthocyanins during processing and digestion is challenging yet essential for functional food

applications. As a result, tailored processing strategies are needed to minimize their degradation and maintain product quality.

2.5.4 Túró Rudi

Túró Rudi is a dairy dessert popular in Central and Eastern European countries, including Hungary. The curd base of Túró Rudi is formed by lactic acid bacterial coagulation of milk (similar to cottage cheese production) in addition to the use of the renneting process. The resulting curd is mixed with cream, generating a sweeter taste and creamy texture to the curd. Túró Rudi is considered a hand-made product which involves three basic steps—milk pre-treatment, Túró making, mixing, forming, and packaging. The high total solid content in the curd-based core (46%-51%) is an important determinant of the final product texture (Csanádi et al., 2016). Research indicates that the stability of Túró Rudi is highly dependent on the pH of the curd filling (Csanádi et al., 2016; Januś et al., 2021). High bacteria counts and the presence of yeast-like microorganisms are monitored as critical quality factors in commercial curd snacks. Standard nutritional profiles for the "Natural" version typically indicate 11 g of protein and 16 g of fat per 100 g, with the carbohydrate content (~36 g) largely dominated by added sugars (Januś et al., 2021). Being a dairy-based snack with a low pH environment, Túró Rudi can be considered as an optimal vehicle for the delivery of phenolic acids such as anthocyanins to the human body (Helal et al., 2022).

2.6 Encapsulation of plant extracts rich in polyphenols

Polyphenols' health benefits can be gained only if they are well absorbed into the human body; therefore, "bioavailability" is a very important concept. When incorporated into food in their free form, polyphenols often interact with macromolecules in the food matrix (Liu et

al., 2016; Alongi et al., 2019). Recent studies highlight the importance of protecting polyphenols from thermal and mechanical stress by using food-grade, biodegradable shell materials before supplementing foods. Preventing the loss of polyphenol activity after oral intake is more challenging, so it's important to explore the most effective technologies to preserve polyphenols and maximize their health benefits. Nevertheless, when phenolic compounds are incorporated into food in their free forms, their bitterness and astringency may negatively affect consumer perception (Bessa-Pereira et al., 2021). On the other hand, powdered plant extracts containing particles >25 microns may induce an unfavorable texture, i.e., sandy mouthfeel (Bessa-Pereira et al., 2021). Therefore, to mask the unfavorable taste of polyphenols, stabilize them to protect their structure through the digestive process, increase their bioavailability, and direct them to specific areas in the human body, this challenge may be addressed by the ENC technology that locks phenolic compounds in wall materials to enhance their stability and antioxidant activity (Nedovica et al., 2011). Specifically, protecting the core material from external factors such as light, moisture, and oxygen helps control the release of encapsulated powder and extends its shelf life. For example, ENC with chitosan nanoparticles, β-cyclodextrin, or yeast cell matrices has been shown to preserve CGA's antioxidant activity and allow its gradual release under gastric conditions (Nallamuthu et al., 2015; Shi et al., 2007). Nallamuthu et al. (2015) showed that CGA-loaded chitosan nanoparticles retained antioxidant activity and thermal stability up to 150˚C. Furthermore, advanced ENC techniques, such as nano-ENC, which reduces particle size to less than 200 nm, enable targeted delivery of phenolic compounds to specific organs (Tylkowski & Tsibranska, 2016). Additionally, ENC

improves water solubility, physicochemical stability, and absorption of polyphenols in the gut, while also masking unpleasant flavors (Lomovskiy et al., 2020; Quintriqueo-Cid et al., 2024). Numerous global studies on the ENC of polyphenols have explored various technologies, including spray-drying, coacervation, liposomes, and nanoparticles, using biodegradable polymers. Typically, most ENC methods involve dehydration because polyphenol-rich plant extracts are liquids (Lomovskiy et al., 2020; Nguyen et al., 2021).

An extensive search of existing publications on the ENC of roselle calyx revealed that spray-drying ENC is the most studied approach so far, and most studies have focused mainly or exclusively on anthocyanins (Nguyen et al., 2021; Millinia et al., 2024), underscoring the need to develop a freeze-drying ENC approach for heat-sensitive phenolics in roselle calyx extracts. Spray-drying ENC is widely practiced because it produces high-throughput free-flowing powders with good storage stability at a low production cost. However, spray-drying method can cause thermal stress, which degrades heat-sensitive phenolic compounds during the process. Freeze-drying ENC, which preserves the functionality of phenolics by minimizing thermal and oxidative stress, is a reliable solution for this drawback of spray-drying (Szpicer et al., 2025). Moreover, the freeze-drying method is simpler and more direct than other ENC methods, as it involves fewer steps than complex methods, such as coacervation, fluidized bed coating, and extrusion (Pudziuvelyte et al., 2020). Freeze-drying ENC includes three main steps—preparation and homogenization of emulsions, freezing, and drying (primary and secondary drying/desorption) (Carpena et al., 2021)—and is therefore simpler than many other ENC methods. However, selecting a coating

material that maximizes the retention capacity and stability of phenolic compounds within the matrix becomes paramount in ENC. Before freeze-drying, emulsifying phenolic-rich plant extracts with coating materials significantly affects ENC efficiency, powder properties, and the storage stability of the core materials (Stabrauskiene et al., 2024). The most commonly used coating materials in freeze-drying ENC include carbohydrates or polysaccharides (e.g., MD, cyclodextrin, chitosan, modified starches), hydrocolloids (e.g., xanthan gum, GA, sodium alginate), and proteins (e.g., gelatin, whey protein isolate, soy protein isolate). For lipophilic phenolic substances, phospholipid- or wax-based excipients are used (Carpena et al., 2021; Pudziuvelyte et al., 2020). Jang and Koh (2024) found that encapsulating anthocyanins with polysaccharides, such as MD, carboxymethyl cellulose, GA, or xanthan gum, enhances retention during digestion.

MD is effective in freeze-drying ENC because it offers high water solubility, low viscosity, and excellent film-forming properties. GA complements MD with its high water solubility, low viscosity, high emulsifying capacity, and high surface activity. Both MD and GA are neutral in taste and aroma. Therefore, combining MD and GA is widely used in recent ENC studies (Lomovskiy et al., 2020; Pudziuvelyte et al., 2020; Kurniati et al., 2025). In a study, Kurniati et al. (2025) found that a 1:4 of MA:GA formulation provided an excellent balance between phenolic retention capacity and physical properties when microgreen kangkong extract was freeze-dried and encapsulated. However, the authors found that increasing GA content increased the water content of encapsulated powder, which was challenging to achieve. According to Todorović et al. (2022), using MD and GA at a 1:1 ratio as a wall material

in lyophillized bilberry extract resulted in high phenolic and TMA contents and antioxidant activity. Additionally, previous studies emphasize the importance of the phenolic extract-to-core material ratio, as it directly affects the drying yield and ENC efficiency. Accordingly, Çam et al. (2014) summarized that maintaining a 1:1 or 1:3 phenolics-to-core material ratio provided improved ENC efficiency. Overall, these findings emphasize the importance of optimizing the wall material composition and its balance with the phenolic extract to facilitate encapsulating target phenolic compounds in the chosen plant material effectively. Additionally, it is crucial to consider the solvent used to extract phenolic compounds from the chosen plant material, as interactions between NADES and excipients in ENC may affect key parameters. In a study, Fitri et al. (2020) tested different combinations of MD and GA in freeze dried-encapsulating NADES extracts of green coffee beans. The authors revealed that encapsulated powder with GA was more soggy and had the lowest retention of phenolic compounds, including CGA, owing to increased solution viscosity before freeze-drying, which leads to poor formation of ice crystals. Fitri et al. (2020) demonstrate that for NADES extracts, using combinations of MD and GA provides better results, particularly higher ENC yield with higher phenolic retention.

When encapsulating plant extracts rich in polyphenols, it is important to consider the type of solvent used for the extraction of phenolic compounds as there can be substantial technical hurdles or advantages, mainly stemming from the distinctive physicochemical properties of the solvents themselves, particularly, high-viscosity, and low-volatility than conventional, easily evaporated solvents (Wróbel et al., 2025). For instance, due to the high-viscosity of NADES, excessive amounts of

emulsifying agents or high-shear mixing might be required to create stable emulsions which can be challenging for sensitive phenolic compounds. Additionally, releasing the active ingredient from the solvent would be perplexing owing to the high affinity of NADES to the extracted phenolics. For instance, Wróbel et al. (2025) reported that the high viscosity and low volatility of choline chloride-based NADESs prevent fiber formation under standard electrospinning conditions, although NADESs could enhance the anthocyanin recovery from red cabbage. In contrast, a study by Miao et al. (2024) found that the ENC efficiency of curcumin in NADES-based high internal phase emulsions was always greater than 85%, attributed to the co-crystal formation of curcumin with NADES, enabling better embedding of curcumin in the emulsion's internal phase. However, the literature on encapsulating NADES extracts is minimal, emphasizing the importance of more research on standardizing protocols for the preparation and characterization of ENC systems containing NADES-based plant extracts.

Although ENC serves as a promising strategy to improve the stability of polyphenols, such as CGA and anthocyanins, before incorporating them into dairy systems, encapsulated phenolics may still form interactions with milk proteins, which are complex and depend on various conditions such as the type of protein, pH and processing conditions, and should be considered as they may impact the release and bioavailability of those phenolics. For instance, covalent interactions are known to improve the stability of protein-CGA complexes during ENC, protecting CGA from degradation and enhancing the bioavailability and antioxidant activity. Additionally, these interactions can alter the structure and functionality of proteins, for instance, improve the emulsifying properties (Poojari et al.,

2023). Nevertheless, due to the limited availability of studies on interactions between encapsulated polyphenols and dairy proteins, there is a need to investigate suitable encapsulating materials, optimize process conditions, and test the release mechanism and interactions with dairy matrix components under *in vitro* and *in vivo* conditions for effectiveness. In addition, limitations in bioavailability are noted, particularly *in vitro* studies that do not accurately reflect human conditions, as well as a scarcity of *in vivo* studies that lack essential data on tissue distribution and metabolism (Jang & Koh, 2024; Pop et al., 2023). Future research should explore cost-effective ENC technologies to improve polyphenol bioavailability and investigate co-ENC methods for synergistic effects. Advancements in these technologies are anticipated to enhance functional foods and offer significant health benefits in the coming decades.

3. Materials and methods

3.1 Materials and instruments

3.1.1 Chemicals

For the preparation of extraction solvents, ethanol (≥98%), methanol (≥98%), lactic acid (≥98%), and citric acid (≥98%) were purchased from VWR chemicals (France), hydrochloric acid (≥98%) from Biolab (Hungary), glycerol (≥87%) from Sigma-Aldrich (Hungary) and D-(+)-glucose anhydrate from Riel-deHaën (Germany). For the preparation of buffer solutions, sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \times H_2O$), sodium acetate (CH_3COONa), acetic acid (CH_3COOH), and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$) were obtained from Merck (Germany). For the phenolic assays, Folin-Ciocalteu reagent, potassium chloride (KCl), and standard gallic acid (≥95%) were purchased from Merck (Germany); anhydrous sodium carbonate from

Riel-deHaën (Germany) and DPPH, L-ascorbic acid (≥98%), aluminum chloride, and standard quercetin (≥95%) from Sigma-Aldrich (Hungary).

For the HPLC measurements, standard 5-CQA (≥95%), and caffeine (≥95%) were obtained from Merck Science Life Ltd. (Hungary), acetonitrile of HPLC grade from Fisher Scientific (UK), and ortho-phosphoric acid of analytical grade from Lachner (Hungary).

For the ENC purposes, maltodextrin-DE19 and gum arabic were purchased from Natural Nutrition (Hungary), and aerosil from E-biola (Poland).

3.1.2 Plant materials

In this study, three different plant materials were use for the extraction of bioactive compounds: commercial roasted coffee (*Coffea arabica*) from Auchan-France (Bellarom-Cyprus) for the methanolic and water extraction of 5-CQA and caffeine; commercial green coffee (*Coffea arabica*) from Gourmet Kave (Hungary) for the Glu-Glyc-based NADES extraction of phenolics including CGAs; and Commercially dehydrated hibiscus (*Hibiscus sabdariffa* L.) flowers from Natur Tea (Hungary) for the ethanolic extraction of 5-CQA and Glu-Glyc, Glu-CA and Glu-LA-based NADES extraction of phenolics including CGA and anthocyanin and further ENC purposes.

3.1.3 Food ingredients

For the production of the curd base of Túró Rudi, commercial túró (semi-fat:7%, protein:16.2%, carbohydrate:3.7%) was purchased from Spar (Hungary), and icing sugar (which is a carbohydrate source composed primarily of 95-98% sucrose with a 2–5% of starch) was purchased from Diamant (Hungary). For the coating of Túró Rudi, dark chocolate (54.5% cocoa—36.6% total fat, 5.1% protein, total carbohydrates 45.8%) was

purchased from Callebaut (Belgium) and for mixing 100% refined sunflower oil (total fat≥92%) was purchased from Golden Imperial (Hungary).

3.1.4 Equipments

For all the dehydration purposes (plant materials and extracts), a 70M laboratory scale dehydrater (Precision Scientific Inc, USA) was used. For grinding coffee beans and dried roselle calyx, a home-scale coffee grinder (ACG-107B Imetec FrescoAroma, China) was used. For the preparation of hot water extracts of roasted coffee capsules, a KP100 coffee machine (Dolce Gusto, France) was used. For the extraction of phenolics from roselle calyx, a GFL 3032 Shaking incubator (Germany) was applied. Samples were mixed using a ZX3 Velp Scientifica vortex mixer (Italy) and centrifuged using a Z206A Hermle laboratory centrifuge (Germany). To dissolve chemicals during phenolic assays, a UC002BM1 Tesla ultrasonic bath (Prague, Czechoslovakia) was used. For all the mass measurements, a TE214S analytical balance (Sartorius, Germany) was used. For the preparation of NADES mixtures and other dissolving purposes, an MR 3004 magnetic stirrer (Heidolph, Germany) was used. For all the pH measurements, an ORION 370 Thermo Scientific pH meter (USA) was applied. The high-purity deionized water was generated with a Milli-Q SQ 2 series Ultra-pure water system (Germany). To prepare encapsulates, a FD-3-85A-MP freeze dryer (Flexidry FTS Systems, USA) was used. Spectrophotometric assays were performed using a Spectroquant Pharo 100 spectrophotometer (Merck, Germany) and a U-2910 spectrophotometer (Hitachi, Japan) was used for pH stability testing of encapsulates. The instrumental analysis of the 5-CQA and caffeine was performed using an HPLC-PDA system equipped with a 4-line degasser

(DG-2080-54), HPLC pump (PU-980), ternary gradient unit (LG-980-02), autosampler (AS-2055), detector (MD4010), and a column thermostat (Phenomenex TS-130) maintained at 35˚C. ChromNAV software (Jasco, Japan) was used for data acquisition. Nutritional analysis of developed Túró Rudi was performed with a near-infrared rapid dairy analyzer (FoodScan^{TM} 2 FOSS, Denmark).

3.2 Sample preparation and extraction procedures

3.2.1 Preparation of roasted coffee extracts (conventional solvents)

Roasted coffee samples of seven different types (Table 1) were used to determine caffeine and 5-CQA concentrations. Extractions were performed in triplicate using three different extraction procedures, explicitly developed for this thesis. In the first method, 0.25 g of roasted capsule coffee was mixed with 40 mL of 60% (v/v) methanol in a graduated flask. The mixture was homogenized using an ultrasound bath and then heated under reflux for 20 min. In the second method, a coffee capsule of known mass (as given in Table 1) was extracted with 50 mL of boiling water using a coffee machine and subsequently diluted to a final volume of 1000 mL. In the third method, a coffee capsule of known mass (as given in Table 1) was extracted with 300 mL of boiling water using a coffee machine and subsequently diluted to a final volume of 1000 mL.

Table 1 Types of commercial coffee used for the analysis of caffeine and 5-CQA concentrations

Type of coffee	Strength/intensity	Mass of coffee in capsule (g)
Ristretto Espresso	10/12	5.620
Viola Espresso	9/12	5.131
Classico Espresso	4/12	5.053
Espresso Intense	10/12	5.043
Forte Lungo	10/12	5.446
Milano Doce Gusto	8/12	5.896
Espresso Decaffeinated	6/12	5.971

3.2.2 Preparation of ethanolic extracts of roselle calyx

Ethanolic extracts of roselle calyx were prepared using a specifically designed method for this thesis. In all treatments, 1 g of dehydrated hibiscus flowers was used to prepare 100 mL of extract. Hibiscus extracts were prepared in triplicate following a 3^3 factorial design to evaluate the effect of solvent composition, pH, and extraction time on the yield of 5-CQA extracted from hibiscus calyx. The pH was adjusted using 10 mL of the corresponding 0.1 M buffer solution: $NaH_2PO_4 \times H_2O$ with H_3PO_4 for pH 2, CH_3COONa with CH_3COOH for pH 4.5, and $NaH_2PO_4 \times H_2O$ with $Na_2HPO_4 \times 2H_2O$ for pH 7.0. All samples were heated under reflux at the

solvent's boiling point for the specified extraction time, with timing starting once boiling commenced (Figure 3) (Table 2).

Figure 3 Roselle calyx extracted with 25%, 50% and 75% (v/v) ethanol at varying pH—2.0, 4.5, and 7.0 (Left: extracts after heat refluxing, Right: extracts after centrifuging)

Table 2 Experimental conditions (3^3 factorial design) for the extraction of 5-CQA from roselle calyx

Treatment ID	Time (min)	Ethanol concentration (%)	pH Value
T1	20	25	2.0
T2	20	25	4.5
T3	20	25	7.0
T4	20	50	2.0
T5	20	50	4.5
T6	20	50	7.0
T7	20	75	2.0
T8	20	75	4.5
T9	20	75	7.0
T10	40	25	2.0
T11	40	25	4.5
T12	40	25	7.0
T13	40	50	2.0
T14	40	50	4.5
T15	40	50	7.0
T16	40	75	2.0
T17	40	75	4.5
T18	40	75	7.0
T19	60	25	2.0
T20	60	25	4.5
T21	60	25	7.0
T22	60	50	2.0
T23	60	50	4.5
T24	60	50	7.0
T25	60	75	2.0
T26	60	75	4.5

Treatment ID	Time (min)	Ethanol concentration (%)	pH Value
T27	60	75	7.0

A response surface model (RSM) using a box-Behnken design was employed to evaluate the relationship between three independent variables (pH, ethanol concentration, and extraction time) coded at three levels (-1, 0, and +1), and the dependent variable, defined as the area of the 5-CQA peak in the chromatogram. The model also aimed to identify the optimal levels of the independent variables to maximize the 5-CQA peak area. Quantification of 5-CQA in each extract was performed using the HPLC method described below in Section 3.3.2.

3.2.3 Preparation of natural deep eutectic solvent extracts of green coffee beans

NADES extracts of green coffee beans were prepared following the method described by Sik et al. (2024), with slight modifications. First, green coffee beans were dehydrated at 40-45˚C for 6 hours and subsequently ground to a fine particle size of 400-500 μm. Glucose and glycerin were inititally weighed in a 1:1 M ratio, and high-purity deionized water equivalent to 50% (w/w) of the glucose and glycerin mixture was measured (higher proportion of water was added to increase the polarity of final mixture), and heated at 90˚C with stirring 500 rpm for approximately 3 hours, using 10 mL of previously measured high-purity deionized water, until a clear, viscous liquid formed. The remaining high-purity deionized water was subsequently added to reduce viscosity, and the resulting solvent was cooled to room temperature before being used for extraction. A weighed sample (0.25 g) of powdered green coffee bean was mixed with 5 mL of Glu-Glyc-based NADES and placed in a shaking incubator at 30±2˚C and 150 rpm for 30, 60, or 90 min. To evaluate the effect of higher-

temperature, short-time extraction, a 50˚C temperature was applied at 150 rpm for 30 min, following the same procedure. After extraction, the samples were transferred to centrifuge tubes and centrifuged at 6000 rpm for 10 min. After the supernatants were diluted fivefold with high-purity deionized water, the filtered (with a 13 mm hydrophilic 0.22 μm filter) samples were used for spectrophotometric and HPLC analyses. Solvent volume and dilution were selected after pre-trials composed of 5 mL volume with twofold dilution, 10 mL volume with fivefold dilution and 20 mL volume with tenfold dilutions. To facilitate comparisons, water extracts and 50% (v/v) aqueous ethanolic extracts were prepared under the same extraction conditions, based on the finding by Tripathi et al. (2025) indicating 50% (v/v) aqueous ethanol with medium polarity provides superior antioxidant capacity and polyphenolic yields compared to higher or lower polarity mixtures.

3.2.4 Preparation of natural deep eutectic solvent extracts of roselle calyx

NADES extracts of roselle calyx were prepared following the method described by Sik et al. (2024), with slight modifications. Three different NADES mixtures were prepared by combining the following constituents: 1:1 M ratio of Glu-Glyc; 1:1 M ratio of Glu-CA; and 1:5 M ratio of Glu-LA. Each mixture was initially weighed. High-purity deionized water equivalent to 50% (w/w) of each mixture (a higher proportion of water was added to increase the polarity of the final mixture) was also measured. Then, each mixture was separately heated at 90˚C with stirring at 500 rpm for approximately 3 hours, using 10 mL of previously measured high-purity deionized water, until a clear, viscous liquid formed. The remaining high-purity deionized water was subsequently added to reduce viscosity,

and the resulting solvent was cooled to room temperature before use for extraction.

Powdered roselle calyx (particle size<500 μm) was combined with the three different NADES mixtures separately in Erlenmeyer flasks to achieve a solid-to-solvent (w/w) ratio of 1:20. Then, samples were placed in a shaking incubator set to two different temperatures: 30˚C and 50˚C, at 150 rpm for 30 min. The extraction time was fixed at 30 min. In parallel, a comparative experiment was performed using 80% (v/v) ethanol and 80% (v/v) ethanol acidified with 0.1% hydrochloric acid, as reference conventional organic solvents based on previous roselle calyx-based experiments (Singh et al., 2021; Manjula et al., 2022), with slight modifications. After extraction, the samples were transferred to centrifuge tubes, and centrifuged at 6000 rpm for 10 min. After the supernatants being diluted fivefold with high-purity deionized water, the filtered (with a 13 mm hydrophilic 0.22 μm filter) samples were used for spectrophotometric and HPLC analysis.

3.3 Developing an HPLC method to analyze 5-CQA concentration in plant extracts

3.3.1 Preparation of 5-CQA standard

The stock solution of 5-CQA (1 mg/mL) was prepared by dissolving 50.0 mg of 5-CQA in 50 mL of ethanol. Working standard solutions with concentrations ranging from 5 to 50 μg/mL were prepared by diluting the stock solution with high-purity deionized water. Each working standard solution was transferred into 1.5 mL HPLC vials for analysis after filtering with a 13 mm hydrophilic 0.22 μm filter.

3.3.2 HPLC analysis of 5-CQA in plant extracts

To quantify 5-CQA in ethanolic extracts of roselle calyx, separate 5-CQA calibration curves were developed by plotting peak area against 5-CQA concentration. The separation was performed using two HPLC columns: an InterSustain Phenyl column (5 μm, 3 mm×150 mm), and a Synergi Polar column (4 μm, 4.6 mm×250 mm), following a predetermined isocratic procedure, specifically designed for this thesis with considerable modifications to the previous studies (Deineka et al., 2019; Jeon et al., 2019; Ludwig et al., 2024). The mobile phase consisted of 7% (v/v) acetonitrile with 93% (v/v) high-purity deionized water containing 0.1% (v/v) H_3PO_4 for the Phenyl column, and 10% (v/v) acetonitrile with 90% (v/v) high-purity deionized water containing 0.1% (v/v) H_3PO_4 for the Polar column. The flow rate and injection volume were maintained at 1.2 mL/min and 2 μL, respectively, and the wavelength was set to 325 nm to monitor the chromatographic profile. For the methanolic/water extracts of roasted coffee and NADES extracts of green coffee beans and roselle calyx, the 5-CQA calibration curve developed with the InterSustain Phenyl column was used following the respective procedure described above. Method validation was performed by evaluating the calibration range, linearity (R^2), repeatability, standard error (SE), limit of detection (LOD), and limit of quantification (LOQ), following the procedure described by Chaowuttikul et al. (2020). Specificity was assessed through a peak purity test, comparing the spectra of all chromatographic peaks with the reference spectrum at the peak apex. Accuracy was evaluated using the recovery method, with percent recoveries determined by spiking 100 μL of 1 mg/mL 5-CQA standard solution into 5 mL of each sample.

Due to the unavailability of 3-CQA and 4-CQA standards in our laboratory, the quantification of 3-CQA and 4-CQA in the NADES extracts of green coffee bean and roselle calyx was performed by multiplying the respective isomer concentration as calculated with the 5-CQA standard curve ($Conc._{isomer}$) by the respective molar extinction coefficients (ε) (5-CQA=1.95, 3-CQA=1.84, 4-CQA=1.80) as previously described by Farah et al. (2005) (Eq. 1). The elution order was identified with literature. Identification of CGA isomer (3-CQA and 4-CQA) peaks was performed following Deineka et al. (2019).

$$Concentration\ of\ CQA\ isomer = \frac{Peak\ area\ of\ 5\text{-}CQA}{1.95} \times \varepsilon\ of\ CQA\ isomer \quad \text{Eq.1}$$

3.4 Developing an HPLC method to analyze caffeine concentration in green and roasted coffee extracts

3.4.1 Preparation of caffeine standard

Similarly, the caffeine stock solution (1 mg/mL) was prepared by dissolving 50.0 mg of caffeine in 50 mL of methanol. Working standard solutions ranging from 5 to 100 µg/mL were prepared by diluting the stock solution with high-purity deionized water. Each working standard solution was transferred into 1.5 mL HPLC vials for analysis after filtering with a 13 mm hydrophilic 0.22 µm filter.

3.4.2 HPLC analysis of caffeine in green and roasted coffee extracts

To quantify caffeine concentration in green coffee and roasted coffee extracts, a caffeine calibration curve was developed by plotting peak area against caffeine concentration. Separation was achieved using an InterSustain Phenyl HPLC column (5 µm, 3 mm×150 mm) following an isocratic procedure. The mobile phase consisted of 7% acetonitrile in 93% high-purity deionized water containing 0.1% H_3PO_4. The flow rate and

injection volume were maintained at 1.2 mL/min and 10 µL, respectively, and the wavelength was set at 275 nm to monitor the chromatographic profile of caffeine. This method was specifically designed for the simultaneous analysis of caffeine and CGA in coffee products with considerable modifications to previous studies (Jeon et al., 2019; Ludwig et al., 2024). Method validation was performed by evaluating the calibration range, R^2, repeatability, SE, LOD, and LOQ, following the procedure described by Chaowuttikul et al. (2020). Specificity was assessed through a peak purity test, comparing the spectra of all chromatographic peaks with the reference spectrum at the peak apex. Accuracy was evaluated using the recovery method, with percent recoveries determined by spiking 100 µL of 1 mg/mL caffeine standard solution into 5 mL of each sample.

3.5 Spectrophotometric analysis of phenolic compounds in plant materials extracted with natural deep eutectic solvents

3.5.1 Total phenolics in green coffee beans and roselle calyx

The TPC of the extracts was measured by the Folin-Ciocalteu method as described by Sik et al. (2024), with some modifications. In brief, 100 µL of each sample was mixed with 1.5 mL of high-purity deionized water, 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent, and 2 mL of sodium carbonate (7.5 g/100 mL) solution. The mixtures were incubated at room temperature for 90 min, and absorbance was measured at 725 nm against a blank. The TPC of each extract was compared to a gallic acid standard curve ($y=1.2929x+0.011$, $R^2=0.999$, 1-1000 µg/mL, SE=0.010 (<1% of the total slope), LOD=0.01 mg/mL, LOQ=0.05 mg/mL). TPCs were expressed as mg of gallic acid equivalents per gram (mg GAE/g) of dry plant material (green coffee beans or roselle calyx).

To build up the calibration curve, a gallic acid stock solution (1mg/mL) was prepared by dissolving 25 mg of gallic acid in 25 mL of methanol. Working standard solutions, ranging from 0.01 to 1 mg/mL, were obtained by diluting the 1 mg/mL stock solution with high-purity deionized water.

3.5.2 Determination of total flavonoid content in roselle calyx

The TFC of the extracts was measured following the method described by Sik et al. (2024), with some modifications. A 500 µL of sample was mixed with 1.5 mL of methanol, 100 µL of 10% (v/v) aluminum chloride, 100 µL of sodium acetate (1 M), and 2.8 mL of high-purity deionized water. The mixtures were allowed to stand in the dark at room temperature for 30 min. The absorbances were measured at 415 nm against the sample blank, which contained roselle extract and all the reagents except aluminum chloride to account for the background reddish hue of the roselle extracts. The TFC of each extract was compared to a quercetin standard curve ($y=0.0088x+0.003$, $R^2=0.999$, 10-200 µg/mL, SE=0.000049, LOD=2.85 µg/mL, LOQ=8.63 µg/mL). TFCs of roselle calyx were expressed as mg of quercetin equivalents per gram of dry plant material (mg QE/g).

Working standard solutions, ranging from 10 to 200 µg/mL, were obtained by diluting the 1 mg/mL stock solution (first prepared by dissolving 25 mg of quercetin in 25 mL of methanol) with high-purity deionized water.

3.5.3 Determination of total monomeric anthocyanin content in roselle calyx

In this study, TMA content was measured in hibiscus calyx extracts using a pH differential method described by Lee et al. (2005). To measure

the TMA content, 0.5 mL of each extract was diluted with 4.5 mL of sodium acetate (0.4 M, pH 4.5) and potassium chloride buffer (0.025 M, pH 1.0), respectively, and incubated for 30 min. Absorbance was measured at 520 and 700 nm versus high-purity deionized water. The difference in absorbance (A) was calculated using Eq. 2.

$$A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5} \qquad \text{Eq.2}$$

Anthocyanin pigment concentration was calculated using Eq.3 and expressed as mg delphinidin-3-sambubioside equivalents per gram of dry plant material (mg DSE/g). A molar absorption coefficient (ε) of 26.900 L/mol/cm and a molecular weight (*MW*) of 597.5 mg/mol were used to calculate the TMA contents. *DF* and *L* represent the dilution factor and path length (internal width of the cuvette), respectively.

$$Anthocyanin\ pigment\ concentration\ \left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L} \qquad \text{Eq.3}$$

3.5.4 Determination of DPPH radical-scavenging activity of roselle calyx

Antioxidant activity of the extracts was measured by the DPPH radical-scavenging activity method, combining the method and equations described by Sik et al. (2024) and Millinia et al. (2024), with some modifications. Each diluted extract (100 µL) was mixed with 3 mL of 0.1 mM methanolic DPPH solution, and the reaction mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against the blank (100 µL of methanol+3 mL of DPPH solution). The % inhibition was calculated using Eq.4 given below.

$$\%\ Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad \text{Eq.4}$$

The % inhibition values (DPPH radical scavenging activity) of each extract were compared to the ascorbic acid standard curve (y=0.0046x-

0.0023, R^2=0.999, 10-200 μg/mL, SE=0.0002, LOD=22.11 μg/mL, LOQ=67.03 μg/mL), and the antioxidant activities were expressed as mg of ascorbic acid equivalents per gram of dry plant material (mg AAE/g). According to the LOD, the first data point appeared to be distinguishable from the background signal, while samples with concentrations below the LOQ tend to be less reliable, showing a limitation in this calibration curve. Therefore, the validity range of the curve is 67.03 to 200 μg/mL. However, as roselle extracts usually fall above 100 μg/mL antioxidant activity, the curve was found to be satisfactory for this study.

To develop the calibration curve, working standard ascorbic acid solutions were prepared ranging from 10 to 200 μg/mL by diluting the stock solution (first prepared by dissolving 25 mg of ascorbic acid in 25 mL of deionized high-purity water). The curve was developed by plotting the % inhibition against ascorbic acid concentration.

3.6 Encapsulation of the natural deep eutectic solvent extract of the roselle calyx

For the ENC, Glu-LA NADES was identified as the best solvent to extract phenolics from roselle calyx. Moreover, lactic acid-based mixture was especially preferred as it provides an acidic environment to the encapsulated powder, which helps to stabilize phenolics of interest and aids in solubilizing polysaccharide-based excipients used in ENC (Sallustio et al., 2024).

First, the Glu-LA (1:5 M, 50% high-purity deionized water w/w) NADES was prepared as described in Section 3.2.4. The roselle calyx extract was then prepared by dissolving powdered roselle calyx in Glu-LA solvent at a 1:20 (w/w) ratio. The mixture was incubated with shaking at 50˚C and 150 rpm for 30 min. After centrifugation at 6000 rpm for 10 min,

the clear supernatant, free from flakes or residues, was used for ENC. Density (g/mL) of the Glu-LA extract of roselle calyx was measured by recording the mass of a 5 mL sample in grams in triplicate, before initiating the ENC procedure.

ENC method for the NADES extract of roselle calyx was distinctly designed for this thesis by making considerable modifications to the methods utilized in previous studies for different plant extracts (Fitri et al., 2020; Todorović et al., 2022). For the excipients, MD and GA were combined in 5 different ratios: A (1:0), B (1:3), C (1:1), D (3:1), and E (0:1). Each 10 g mixture was dissolved in 6 g of high-purity deionized water at 45-50˚C, then mixed with 4 g of extract. The mixture was supplemented with 0.14 g of Aerosil (silicon dioxide) and stirred thoroughly with a glass rod until no clumps were remained. The resulting mixtures were transferred into glass beakers and frozen at -25˚C for 12 hours as a pre-freezing step. Afterthat, the samples were freeze-dried at -80˚C with maintained pressure of 90 mT for 24 hours. The dried materials were ground into a fine powder and stored in tightly sealed bottles under refrigerated (~ 4˚C). A known mass of NADES roselle calyx extract was also freeze-dried separately to determine the solid content of the extract.

The five encapsulated powders, with various combinations of MD and GA, were evaluated for ENC efficiency, drying yield, and moisture content.

3.6.1 Determination of encapsulation efficiency

ENC efficiency was determined as a function of total anthocyanin added and surface anthocyanin, and expressed as a percentage (Eq. 5), following the method described by Millinia et al. (2024), with slight modifications.

$$ENC\ Efficiency\ \% = \frac{Total\ Anthocyanin - Surface\ Anthocyanin}{Total\ Anthocyanin} \times 100 \qquad \text{Eq.5}$$

For the total anthocyanin determination, a 0.75 g sample of each encapsulated powder was crushed with 2 mL of high-purity deionized water using a mortar and pestle, transferred into a test tube, and added with 13 mL of 80% (v/v) ethanol, vortexed for 5 min to liberate all core compounds, and filtered using a 0.45 μm PTFE membrane. Conversely, for the surface anthocyanin determination, 0.75 g of each encapsulated powder was added with 15 mL of 80% (v/v) ethanol, vortex-mixed for 30 s, as a brief washing step, which strictly enforced to selectively dissolve superficial, unencapsulated pigments without inducing core matrix leaching out. Afterthat, the samples were centrifuged at 6000 rpm for 10 min, and the supernatant was used for further analysis after filtering with a 0.45 μm PTFE membrane. Total anthocyanin and surface anthocyanin contents were measured using the pH differential method described in Section 3.5.3.

3.6.2 Drying yield of encapsulated powder

Drying yield of encapsulated powder was calculated (Eq.6) using the final dry weight of the encapsulated powder (DW_{final}) and the total initial weight of all solids (WI). The method was adopted from Fitri et al. (2020).

$$Drying\ Yield\ \% = \frac{DW_{final}}{WI} \times 100 \qquad \text{Eq.6}$$

3.6.3 Moisture content of encapsulated powder

A measured weight of the encapsulated powder (W_i) was heated at 105°C for 30 min until the weight remained constant (W_f), and the dry basis moisture content was calculated as a percentage of the difference between W_i and W_f, divided by the dry weight, following the method described by Fitri et al. (2020).

3.6.4 Determination of functional properties of encapsulated powder

The treatment that demonstrated the highest ENC efficiency was further analyzed for TPC, TMA, and DPPH radical-scavenging activity. For those measurements, samples were prepared similarly to the method followed in Section 3.6.1. More particularly, encapsulated powder was ruptured to get the total TPC, total TMA and total DPPH radical-scavenging activity values, and surface measurements were taken, and the difference in those two measurements was considered to improve the accuracy. TPC, TMA, and DPPH radical-scavenging activities were assessed following the methods described in Sections 3.5.1, 3.5.3, and 3.5.4, respectively.

3.6.5 Determination of powder properties and solubility

To determine the Carr index and Hausner ratio of the selected encapsulated powder (treatment D), bulk and tapped density were measured following the method described by Caliskan & Dirim (2016), with slight modifications. Briefly, the bulk density was calculated by filling a 10 mL graduated cylinder with a 5.0 g sample of encapsulated powder and dividing the mass by the volume. To get the tapped density, the same graduated cylinder was tapped 120 times and the mass was divided by the recorded volume. The Carr index and Hausner ratio were calculated using the equations given below.

$$Carr\ Index = \frac{Tapped\ density - Bulk\ density}{Tapped\ density} \times 100 \qquad \text{Eq.7}$$

$$Hausner\ Ratio = \frac{Tapped\ density}{Bulk\ density} \times 100 \qquad \text{Eq.8}$$

The solubility of the encapsulated powder was expressed as the average solubility time (s) of the powder product and was determined by

completely dissolving 2.0 g of sample in 50 ml of high-purity deionized water at 30±2˚C under continuous stirring using a magnetic stirrer, as described by Caliskan & Dirim (2016). The rehydration behavior of the encapsulated powder was visualized by plotting a time-dependent solubility curve based on the experimental endpoint (time in seconds) using a first-order kinetic model.

3.6.6 Determination of color stability (by spectrophotometric absorbance) at varying pH levels

Color stability of the encapsulated powder was determined by combining the methods described by Milinia et al. (2024) and Wahyuningsih et al. (2017), with modifications. A 0.1 g sample of the encapsulated powder was dissolved in 10 mL of buffer solutions at four pH levels—1.0, 2.0, 4.5, and 6.8. The color intensity of each mixture was measured by absorbance at 535 nm using a Hitachi U-2910 spectrophotometer over three hours. The degradation rate constant (k) was calculated at pH 6.8 using Eq.9, where t, C_0, and C_t stand for the time elapsed, initial concentration, and concentration at the elapsed time (Wu et al., 2018).

$$k = \frac{1}{t} ln \times (C_0/C_t) \qquad \text{Eq.9}$$

3.7 Developing a dairy dessert (Túró Rudi) incorporating freeze-dried encapsulated powder of roselle

3.7.1 Preparation of enriched Túró Rudi

Milk dessert containing roselle extract was explicitly designed for this thesis using commercially available semi-fat curd (7% w/w of fat). Basically, the standard guidelines for “curd sticks/curd snacks” produced by the Hungarian Food Codex Committee (2023) were followed for developing the product. A 250 g portion of curd was creamed and mixed

with 50 g of icing sugar, 1 teaspoon of vanilla extract, 8 g of vanillin sugar, the grated zest of one medium-sized lemon. Encapsulated roselle extract powder was added at three levels (2%, 5%, 9%, w/w) calculated based on the combined weight of the curd basis and the respective mass was replaced with the encapsulated powder. In these formulations, roselle encapsulated powder was measured on a weight basis relative to the combined weight of curd, icing sugar, and vanillin sugar mixture. A control sample was also prepared without any plant extracts. The mixtures were homogenized to achieve a uniform consistency and then shaped into bars of 2 cm thickness. The bars were cut into 4 cm lengths, frozen for 1-2 hours, and subsequently coated with melted dark chocolate (54.5% cocoa) mixed with 2-3 drops of sunflower oil. Produced chocolate-coated bars were stored under frozen conditions at -18˚C until further analysis.

3.7.2 Analysis of antioxidant properties of Túró Rudi fortified with roselle encapsulated powder

First, to defat the Túró Rudi, a 1.0 g sample of Túró Rudi (composed of both the coat and the core) was collected from each treatment (exactly from the middle length of Túró Rudi). The sample was crushed using a mortar and pestle to make the sample composed of both the curd-base and coat homogeneously to achieve a uniform, representative matrix blend, ensuring that subsequent analytical replicates captured a consistent, proportional ratio of both the curd and coat. Afterwards, the paste was mixed with n-hexane (1:10 g/mL) in a beaker with a glass rod and transferred into a Falcon tube for vortexing for five mins. Afterwards, the resulting mixture was centrifuged for 10 mins at 6000 rpm, and the supernatant containing fat was removed. Washing the sample with n-hexane was repeated for the second time, and the remaining solid product

was oven-dried at 40˚C for 45 mins and used for the extraction. For the analytical extraction, aqueous ethanol was selected as it provides the ideal lower viscosity, facilitating the extraction of target phenolics without interfering with spectrophotometric assays, particularly TPC and DPPH assays. Moreover, standardizing methods using aqueous ethanol ensures adherence to validated analytical protocols, enabling reliable comparisons of antioxidant retention with the literature. Accordingly, defatted Túró Rudi (0.75 g) was mixed with 80% (v/v) ethanol at a solid-to-solvent ratio of 1:20 (g/mL) and shaken-incubated (150 rpm, 30˚C) for 30 mins. Post-extraction, samples were centrifuged at 6000 rpm for 10 min, and the supernatants were used to measure TPC, TMA, and antioxidant activity as described previously.

3.7.3 Analysis of nutritional and sensory properties of Túró Rudi

First, the moisture content, salt, protein, fat, and total solid contents of all treatments and the reference sample of Túró Rudi were analyzed using a near-infrared rapid food analyzer. For this analysis, only the core of the Túró Rudi was used, considering the specifications of the food analyzer (limited for dairy matrices). A 20.0±2.0 g sample was evenly spread on a Petri dish and loaded into the food analyzer. Measurements for each tested parameter were generated as w/w%±standard deviation (SD) by the food analyzer.

A sensory evaluation test was conducted with 20 semi-trained panelists (70% male, 30% female within the age group of 23-65 years) to assess the sensory acceptability of developed Túró Rudi samples in terms of visual assessment (9-point hedonic scale), comparison with control (herbal flavor, pink/redness, and texture), and overall preference, using a sensory ballot sheet (appendix 1), which was explained to the panelists

before conducting the test. Particularly, Túró Rudi samples of 10±0.2 mm length were presented to panelists. Overall, this sensory evaluation test was adopted from the methods described in Hoang et al. (2024) and Falcão et al. (2023), with considerable modifications.

3.8 Statistical Analysis

All experiments were conducted in triplicate for improved accuracy, followed by an analysis of variance (ANOVA) using Minitab®19 statistical software. All the experimental results were checked for normality using probability plots and referring to the P-values and Anderson-Darling values. As all data followed normality, for the pairwise comparisons, Tukey's HSD test was used at a 5% significance level.

For the development and validation of the RSM, Minitab®19 statistical software was used. More specifically, ANOVA, analysis of response surfaces, residual plots, model p-value, lack of fit, determination coefficient (R^2) and $R^{2(adj)}$ values, regression equations, and contour and surface plots were performed using the above-mentioned software.

For the sensory analysis, the Friedman test (for preference with a 9-point hedonic scale and perceived difference with the reference) was selected because the sensory data is ordinal and collected using a repeated measures design (where each panelist evaluated every treatment). If the Friedman test results were significant, the Wilcoxon signed-rank test was used for pairwise comparisons at a 95% level of significance. Additionally, the average scores received for each attribute were compared for more practical evaluations. To evaluate the final preference, the chi-square test was used at 95% level of significance.

Other data was analyzed using Microsoft Office Excel. All values were expressed as mean±SD values obtained in experiments performed in triplicate.

4. Results and Discussion

4.1 Quantification of 5-CQA and caffeine in roasted coffee extracts

This section of the study was primarily conducted to quantify the 5-CQA and caffeine in seven of the most popular European roasted coffee types. Methanol was included as one of the solvents due to its reported superior performance compared to other organic solvents in extracting bioactive compounds. In the selected three methods, one was typical hot water extraction, which follows the home-scale coffee-making procedure using a coffee machine. The other method involved a larger volume of water (300 mL) to identify whether larger volumes aid in extracting more bioactive compounds from coffee.

Calibration curves established to measure caffeine and 5-CQA in coffee extracts using the InterSustain Phenyl HPLC column had 9.5±0.01 min and 5.8±0.02 min retention times, respectively. The regression equations indicated strong linearity, with caffeine showing y=38153x and 5-CQA showing y=37989x across the concentration ranges of 5-100 μg/mL and 5-50 μg/mL. Both substances exhibited high correlation coefficients (0.999), indicating a solid correlation between concentration and peak area, with R^2 values exceeding 0.99, confirming the effectiveness of the regression models. LOD and LOQ values for the caffeine curve were 2.5 μg/mL and 7.7 μg/mL, respectively, while those values were 0.69 μg/mL and 2.1 μg/mL for the CGA curve, respectively. Caffeine and CGA curves had SE values of 372.7 and 204.8, respectively (<1% relative error for both curves), indicating higher sensitivity and reliability.

Tables 3 presents caffeine and 5-CQA concentrations in mg per gram of coffee based on the methanolic extraction method.

Table 3 Caffeine and 5-CQA concentrations in commercial roasted coffee extracted with 40 mL of 60% (v/v) methanol

Type of Coffee	$C_{caffeine}$ (mg/g)	$C_{5\text{-CQA}}$ (mg/g)
Ristretto Espresso	13.1 ± 0.23^{c}	2.96 ± 0.64^{f}
Viola Espresso	13.3 ± 0.23^{c}	4.04 ± 0.12^{d}
Classico Espresso	13.0 ± 0.12^{c}	5.83 ± 0.12^{b}
Espresso Intense	14.2 ± 0.12^{b}	3.60 ± 0.63^{e}
Forte Lungo	12.1 ± 0.19^{d}	5.98 ± 0.13^{b}
Milano Doce Gusto	19.1 ± 0.23^{a}	4.81 ± 0.12^{c}
Espresso Decaffeinated	0.75 ± 0.05^{e}	6.32 ± 0.12^{a}

Notes: Values represent mean±SD. Means followed by the same superscripts in the same column are not significantly different ($p>0.05$) at a 0.05 probability level according to Tukey's HSD test.

With methanolic extraction, the Milano Dolce Gusto espresso showed the highest caffeine concentration (19.10±0.23 mg/g), significantly exceeding other coffee types ($p<0.05$), while the highest 5-CQA concentration was observed in Decaffeinated Espresso (6.32±0.12 mg/g). In home brewing method, and hot water extraction with larger water volume also, Milano Dolce Gusto espresso showed highest caffeine concentrations (14.6±0.11 mg/g, and 18.5±0.17 mg/g, respectively) (Table 4). Decaffeinated Espresso showed significantly higher 5-CQA yield with hot water extraction with larger water volume (5.70±0.05 mg/g) ($p<0.05$), exceeding all other espresso types, while both Decaffeinated Espresso (5.20±0.06 mg/g) and Classico Espresso at 5.02±0.05 mg/g ($p>0.05$) yielded highest 5-CQA levels in home brewing method.

Table 4 Caffeine and 5-CQA concentrations in commercial roasted coffee extracted with water

Type of Coffee	$C_{caffeine}$ (mg/g)	Recovery $_{caffeine}$%	$C_{5\text{-}CQA}$ (mg/g)	Recovery $_{5\text{-}CQA}$%
50 mL extract diluted to 1000 mL				
Ristretto Espresso	11.3±0.12^{d}	86.3±1.71	2.41±0.05^{e}	81.2±1.72
Viola Espresso	12.0±0.12^{b}	90.3±1.64	3.56±0.05^{c}	88.2±1.28
Classico Espresso	11.5±0.11^{c}	88.5±1.35	5.02±0.05^{a}	86.0±1.70
Espresso Intense	11.4±0.11^{cd}	80.1±1.05	2.79±0.00^{d}	77.5±1.71
Forte Lungo	10.2±0.18^{e}	84.2±1.82	4.88±0.05^{b}	81.5±1.64
Milano Doce Gusto	14.6±0.11^{a}	76.5±1.81	3.57±0.05^{c}	74.4±1.92
Espresso Decaffeinated	0.68±0.00^{f}	90.0±1.81	5.20±0.06^{a}	82.4±1.90
300mL extract diluted to 1000 mL				
Ristretto Espresso	12.5±0.05^{c}	95.6±1.57	2.75±0.05^{f}	92.8±1.81
Viola Espresso	12.7±0.06^{c}	96.1±1.51	3.72±0.01^{d}	92.1±1.99
Classico Espresso	12.1±0.05^{d}	93.6±1.00	5.29±0.05^{b}	90.7±1.75
Espresso Intense	13.3±0.11^{b}	93.7±1.05	3.29±0.01^{e}	91.6±1.89
Forte Lungo	11.2±0.05^{e}	92.3±1.40	5.39±0.05^{b}	90.1±1.47
Milano Doce Gusto	18.5±0.17^{a}	97.3±1.57	4.57±0.05^{c}	95.0±2.30
Espresso Decaffeinated	0.74±0.00^{f}	98.3±2.10	5.70±0.05^{a}	90.1±1.70

Notes: Values represent mean±standard error. Means followed by the same superscripts in the same column under the same extraction method are not significantly different at a 95 % confidence level according to Tukey's HSD test. Recover% was calculated compared to the methanolic extraction method.

Overall, these results indicate that Milano Dolce Gusto coffee consumers intake a much higher dose of caffeine per gram of coffee, which is a critical factor for those monitoring daily caffeine limits (typically 400 mg/day for adults) (Wierzejska & Gielecińska, 2024), while the decaffeination process used in manufacturing decaffeinated espresso used in this study preserved or concentrated these beneficial phenolic compounds while removing the caffeine.

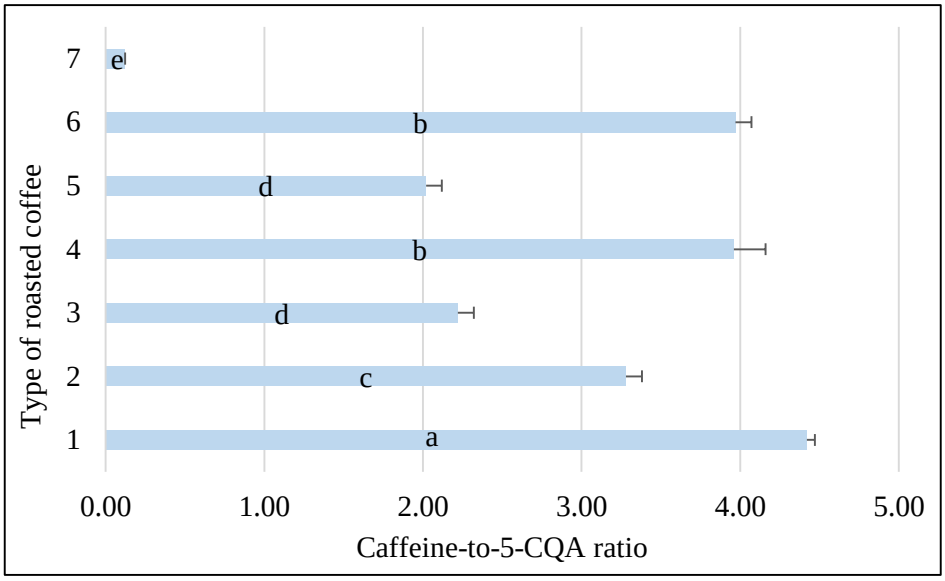
With methanolic extraction, caffeine extracted from various espresso types (except decaffeinated espresso) averaged 14.13±0.20 mg/g, outperforming water extraction methods (11.83±0.17 mg/g and 13.38±0.14 mg/g). For Decaffeinated Espresso, caffeine concentrations were 0.75±0.05 mg/g (methanolic extraction), 0.68±0.00 mg/g (water extraction with home-scale brewing), and 0.74±0.00 mg/g (water extraction with 300 mL of water). Methanolic extraction yielded an average of 4.53±0.30 mg/g of 5-CQA from all espresso types except Decaffeinated Espresso, compared to 3.71±0.00 mg/g and 4.16±0.01 mg/g with water (home-scale brewing and extraction with 300 mL of water). In Decaffeinated Espresso, the concentration of 5-CQA was higher in methanolic extracts (6.32±0.12 mg/g) than in aqueous extracts (5.20±0.06 mg/g and 5.70±0.05 mg/g). These results indicate that hot water extraction has reduced the 5-CQA yields more prominently compared to caffeine yields, attributed to the 5-CQA's considerably lower solubility in water compared to caffeine in water (Jeon et al., 2019). Moreover, there can be a thermal degradation effect on 5-CQA, as hot water was used for the extraction. The methanolic extraction yielded the highest concentrations of caffeine and 5-CQA due to their greater solubility in organic solvents, according to Rojas-González et al. (2022). Recovery rates for caffeine

from hot water methods were 77-90% (home-scale brewing) and 92-98% (water extraction with 300 mL water), while 5-CQA recovery rates were 74-88% (home-scale brewing) and 90-95% (extraction with 300 mL of water), compared to methanolic extraction (Table 4). Thus, infusions made with 300 mL of hot water are more effective in extracting caffeine and 5-CQA than those with home-brewing method with 50 mL.

Roasting significantly affects the caffeine-to-5-CQA ratio in coffee, as roasting decreases 5-CQA content while caffeine remains relatively stable. The caffeine-to-5-CQA ratio being a reliable marker for the degree of roasting, Ristretto Espresso indicates the highest roasting degree among the tested coffee types in this study. Health-wise, coffees with a lower caffeine-to-5-CQA ratio are considered more beneficial, indicating a higher concentration of beneficial CGA relative to caffeine's potential negative effects, such as anxiety and sleep disturbances (Jeon et al., 2019). Moderately consumed caffeine (<400 mg/day) is beneficial, but excessive intake poses health risks. In this study, the caffeine-to-5-CQA ratios for the tested coffee types ranged from 0.12±0.00 to 4.42±0.05, as determined by methanolic extraction (Figure 4). Accordingly, the study identifies Milano Dolce Gusto as a high-stimulant choice and Decaffeinated Espresso as a high-antioxidant choice. The data prove that Decaffeinated

Espresso retained significantly higher levels of health-promoting 5-CQA than its caffeinated counterparts.

Figure 4 Caffeine-to-5-CQA ratios in commercial roasted coffee types extracted with 60% (v/v) methanol. 1: Ristretto Espresso, 2: Viola



espresso, 3: Classico Espresso, 4: Espresso Intense, 5: Forte Lungo, 6: Milano Dolce Gusto, 7: Decaffeinated Espresso.

Notes: Values followed by the same letter are not significantly different at a 95 % confidence level according to Tukey's HSD test. Letters are assigned from a to e in descending order of the ratios. Error bars represent the SD of means.

4.2 Determination of 5-CQA concentration in ethanolic extracts of roselle calyx

4.2.1 HPLC conditions for the separation of 5-CQA

According to the literature, in HPLC, different chemicals such as formic acid and phosphoric acid are used in the aqueous phase to improve the resolution, control ionization, and reduce peak tailing of compounds of interest (Chaowuttikul et al., 2020). In this study, 7% acetonitrile and 0.1% H_3PO_4 (InertSustain Phenyl column) and 10% acetonitrile and 0.1%

H_3PO_4 (Synergi Polar column) with an isocratic program (which showed good resolution (>1.5) and symmetric peak shapes) were selected to separate 5-CQA in hibiscus. The column temperature was held at 35˚C for the duration of the analysis to improve the retention time precision (<1% residual standard deviation RSD%). In HPLC, the identification of compounds in herbal infusions depends on the retention time and UV-light spectral characteristics of the chromatographic peak of the standard solution. According to the literature, hydroxycinnamic acids have the maximum wavelength in the 270-360 nm range (Yilmaz and Kolak, 2017). By comparing the UV spectra of standard 5-CQA at varying wavelengths, 325nm wavelength was identified as the optimum wavelength to detect 5-CQA, which was supported by Farah et al. (2005).

Calibration curves for 5-CQA, based on HPLC analysis with InertSustain Phenyl and Synergi Polar columns, obtained in the form of y=ax+b, demonstrated good linearity between concentration and peak area within the tested range, with regression equations of y=7364x-3495 (InertSustain Phenyl column) and y=4859x-3423 (Synergi Polar column) are depicted in Figure 5.

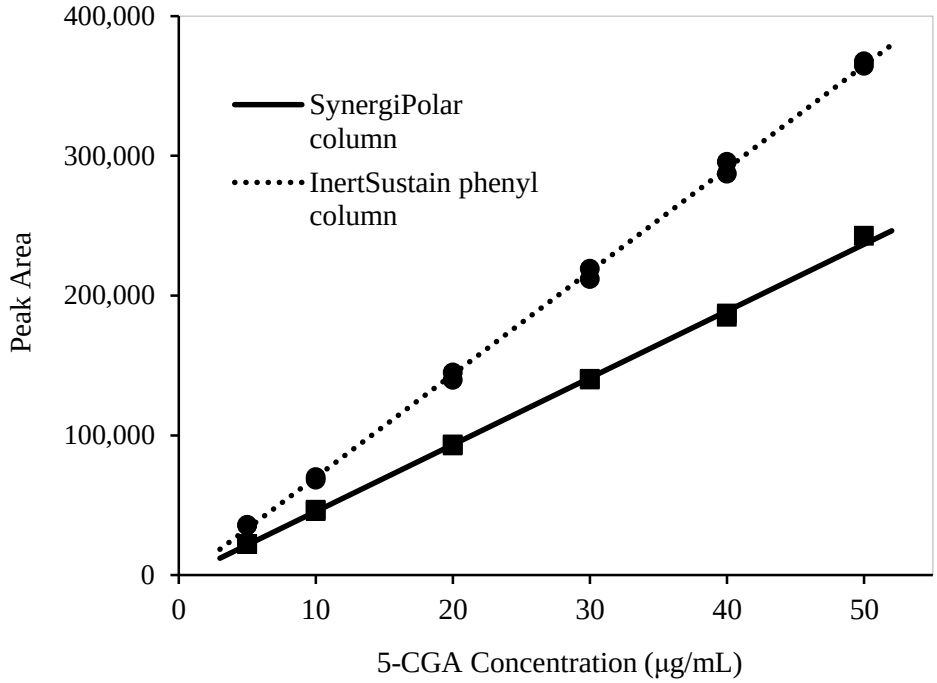


Figure 5 Calibration curve for 5-CQA concentration developed for the two RP HPLC columns: InertSustain Phenyl and Synergi Polar

The retention times for standard 5-CQA at 325 nm were 5.69±0.01 min (InertSustain Phenyl column) and 12.1±0.01 min (Synergi Polar column). The high correlation coefficient values (R_{Phenyl}=0.999, R_{Polar}=0.999) denote an excellent correlation between 5-CQA concentration and peak area. Similarly, R^2 values greater than 0.99 for both columns indicate that the regression model follows linearity and fits the observed data well. An analytical method is considered acceptable when the R^2 value is 0.99 or above (Chaowuttikul et al., 2020). The model p-values for Phenyl and Polar columns were 0.007 and 0.022, repectively, while the lack of fit values for both columns were 0.2, showing that this model can be used for further studies. The SE for Phenyl and Polar

columns were 60 and 65 (1% of the total slope for both columns), revealing that the calibration curves are highly consistent. The LOD values for Phenyl and Polar columns were 1 μg/mL and 2 μg/mL, while the LOQ values were 4 μg/mL and 7 μg/mL, respectively.

4.2.2 Optimization of the extraction conditions and quantification of 5-CQA in ethanolic extracts of roselle calyx

In quantification, it was confirmed that the spectral peaks for the hibiscus extracts were in line with the spectra of standard 5-CQA, which were further confirmed by spiked tests. Recovery percentages calculated with spiked samples were within the acceptable range of 80-120% (105.7% and 105.6% for InertSustain Phenyl and Synergi Polar columns, respectively). The RSD% values for the spiked samples were 2% and 3% for InertSustain Phenyl and Synergi Polar columns, respectively, indicating the accuracy and repeatability of the tested RP-HPLC methods for the quantification of 5-CQA in hibiscus samples.

Figures 6 and 7 show representative base-peak chromatograms of T2 hibiscus extract analysed using two HPLC columns. In the Phenyl column, peak 2 was identified as 5-CQA at 325 nm, while in the Polar column, it appeared as peak 3.

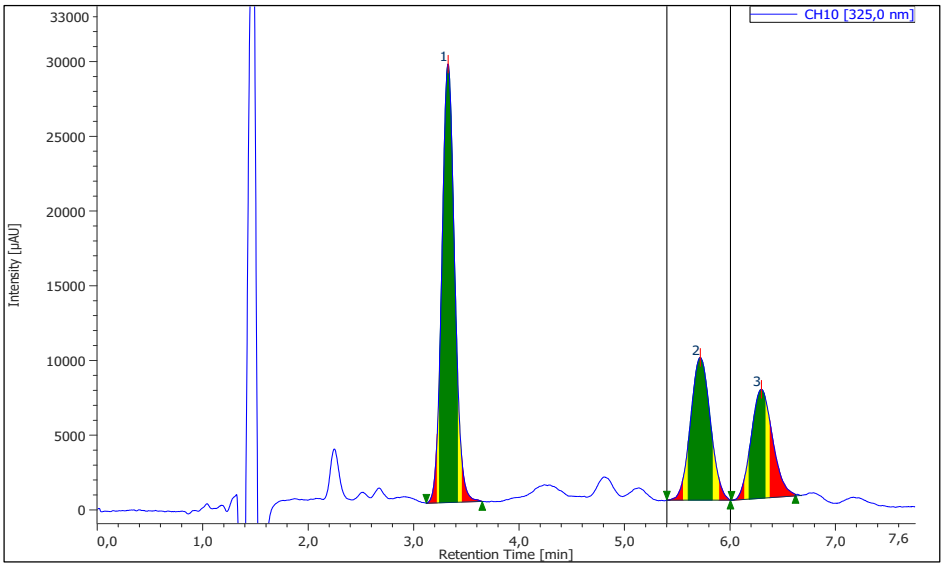


Figure 6 Separation of CGAs with a mobile phase containing 7% acetonitrile and 0.1% H_3PO_4 on InertSustain Phenyl stationary phase at a detector wavelength of 325 nm and 1.2 mL/min flow rate. Peaks 1, 2, and 3 represent 3-CQA, 5-CQA, and 4-CQA, respectively. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.

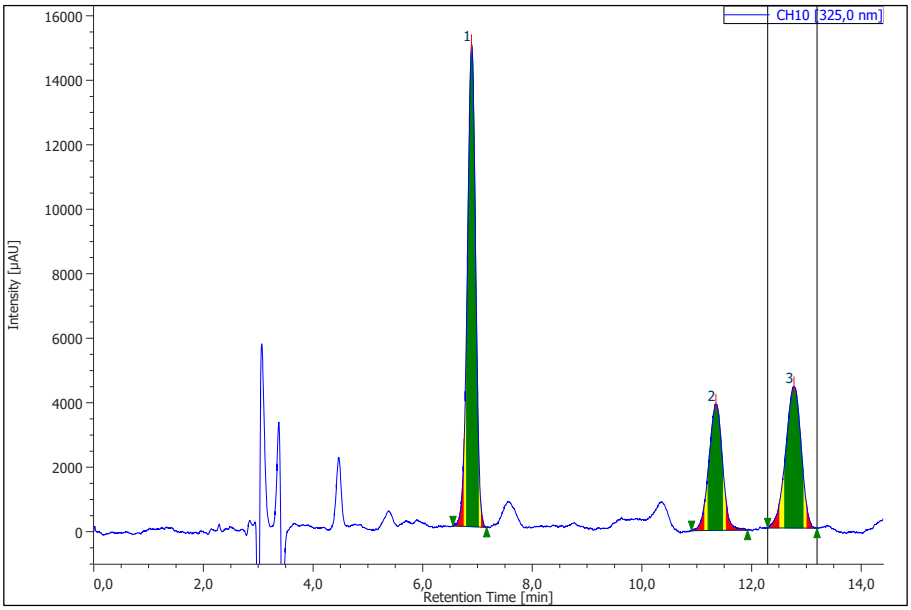


Figure 7 Separation of CGAs with a mobile phase containing 10% acetonitrile and 0.1% H_3PO_4 on Synergi Polar stationary phase at a detector wavelength of 325 nm and 1.2 mL/min flow rate. Peaks 1, 2, and 3 represent 3-CQA, 4-CQA, and 5-CQA, respectively. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.

The extension of the retention times of 5-CQA in the Polar column compared to the Phenyl column can be due to the improved Polar interactions offered by the Polar column. In particular, as 5-CQA has a spatial configuration that makes its aromatic caffeoyl ring completely accessible to the stationary phase with ether-linked phenyl groups, 5-CQA is held back on the column longer than 3-CQA and 4-CQA, causing it to elute last as the 3rd peak. Peak purity was calculated to determine the specificity of the method. Peak purity levels for 5-CQA were 81.28% and 79% for the Phenyl and Polar columns, respectively, indicating potential

impurities. Correlation coefficients with a 50 μg/mL standard were 0.991 and 0.997 for the two columns, endorsing their effectiveness in separating 5-CQA in hibiscus extracts.

As the concentration measured by the Polar-RP column increases, the Phenyl column shows a nearly identical increase (Figure 8), suggesting a positive correlation between the two columns.

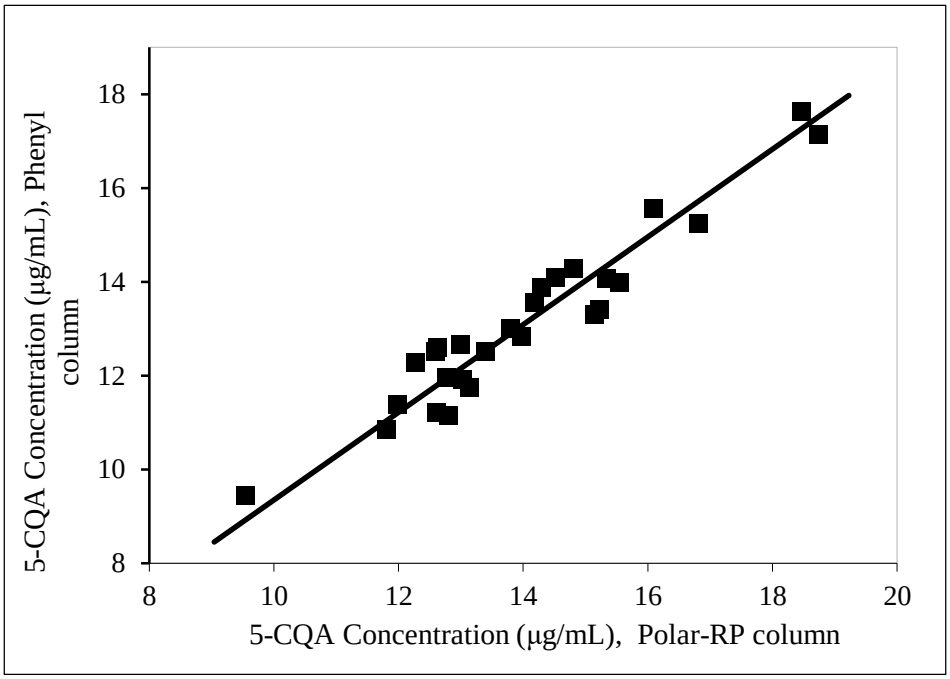


Figure 8 Correlation of the 5-CQA concentration in roselle calyx extracts analyzed with the two RP-HPLC columns: InertSustain Phenyl column *vs.* Synergi Polar column

The determination coefficient of 0.999 and slope of 0.935 demonstrated that there is no significant systematic bias between the two RP-HPLC columns as they are measuring the 5-CQA concentration in roselle calyx extracts consistently, although the Phenyl column yields were generally lower than those of the Polar-RP column. Regression analysis

indicates that either column could likely be used for this specific analysis with very similar results. However, comparative performance (paired T-test) revealed a statistically significant difference ($p<0.05$) between the two columns ($Polar_{mean}$=14.16±2.10 µg/mL *vs.* $Phenyl_{mean}$=13.01±1.83 µg/mL), suggesting that the Polar column appears to provide higher recovery or sensitivity for 5-CQA in the ethanolic extracts of roselle calyx.

The experimental results for 5-CQA concentrations in hibiscus extracts followed normal distribution based on the residual plots generated for both columns (Figure 9) and showed values ranging from 9.4 to 17.6 µg/mL with the InertSustain Phenyl column and 9.5 to 18.5 µg/mL with the Synergi Polar column (Table 5). The highest yields were observed in the T2 extraction (17.6 µg/mL with InertSustain Phenyl and 18.5 µg/mL with Synergi Polar), which used 25% ethanol, a pH 4.5 buffer, and a 20-minute extraction time. This was statistically similar to T5, which had 50% ethanol, pH 4.5, and a 20-minute extraction time ($p>0.05$).

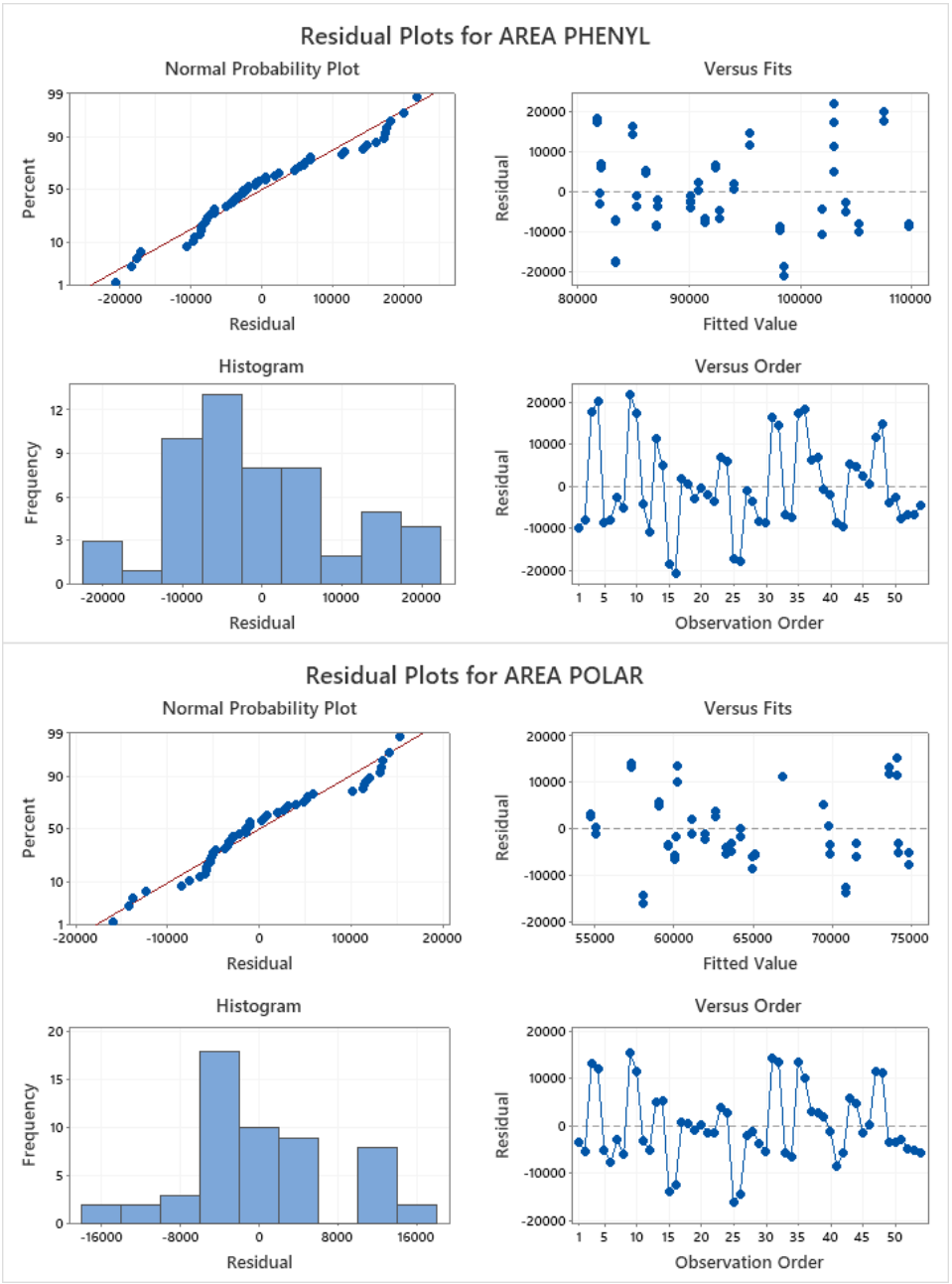


Figure 9 Residual plots for the peak area of InertSustain Phenyl column (up) and Synergi Polar column (down) according to the RSM

Table 5 Concentration of 5-CQA detected in roselle calyx extracts (HPLC analysis with InertSustain Phenyl and Synergi Polar columns)

Treatment ID	Average 5-CQA concentration±SD (μg/mL)—InertSustain Phenyl Column	Average 5-CQA concentration±SD (μg/mL)—Synergi Polar Column
T1	13.6 ± 0.16^{def}	14.2 ± 0.28^{fghi}
T2	17.6 ± 0.24^{a}	18.5 ± 0.18^{a}
T3	14.3 ± 0.07^{cd}	14.8 ± 0.37^{defg}
T4	14.1 ± 0.23^{d}	14.5 ± 0.44^{defg}
T5	17.1 ± 0.43^{a}	18.7 ± 0.55^{a}
T6	13.3 ± 0.63^{defg}	15.1 ± 0.30^{cdef}
T7	15.6 ± 0.62^{b}	16.1 ± 0.03^{bc}
T8	11.2 ± 0.23^{lm}	12.6 ± 0.20^{lmno}
T9	13.4 ± 0.13^{defg}	15.2 ± 0.02^{cdef}
T10	11.4 ± 0.25^{klm}	12.0 ± 0.20^{no}
T11	12.0 ± 0.16^{ijk}	12.8 ± 0.00^{klmno}
T12	13.9 ± 0.09^{de}	14.3 ± 0.18^{efgh}
T13	9.4 ± 0.05^{n}	9.5 ± 0.25^{p}
T14	11.7 ± 0.24^{jklm}	13.1 ± 0.15^{ijklm}
T15	11.1 ± 0.03^{lm}	12.8 ± 0.23^{klmno}
T16	14.1 ± 0.18^{d}	15.3 ± 0.12^{cde}
T17	10.8 ± 0.05^{m}	11.8 ± 0.11^{o}
T18	14.0 ± 0.08^{de}	15.5 ± 0.48^{cd}
T19	12.5 ± 0.07^{ghij}	12.6 ± 0.06^{lmno}
T20	12.5 ± 0.13^{ghij}	13.4 ± 0.45^{hijkl}
T21	12.6 ± 0.09^{fghij}	12.6 ± 0.39^{jlmno}
T22	12.8 ± 0.07^{fghi}	14.0 ± 0.15^{ghij}
T23	13.0 ± 0.19^{efgh}	13.8 ± 0.25^{ghijk}

Treatment ID	Average 5-CQA concentration±SD (µg/mL)—InertSustain Phenyl Column	Average 5-CQA concentration±SD (µg/mL)—Synergi Polar Column
T24	15.2 ± 0.30^{bc}	16.8 ± 0.02^{b}
T25	12.3 ± 0.12^{hijk}	12.3 ± 0.03^{mno}
T26	11.9 ± 0.09^{ijkl}	13.0 ± 0.26^{jklmn}
T27	12.7 ± 0.29^{hijk}	13.0 ± 0.07^{jklmn}

When the area of the 5-CQA peak was analyzed with the Phenyl column, linear extraction variables showed no significant effects ($p>0.05$), while time with ethanol concentration was the only significant two-way interaction ($p<0.05$). In quadratic terms, the effects of time and pH were significant ($p<0.05$). When analyzed with Polar column, pH emerged as the only significant linear extraction variable ($p<0.05$), with a significant interaction between time and ethanol concentration. Quadratic effects of time and pH were also significant ($p<0.05$) (Table 6).

Table 6 ANOVA for the polynomial RSM of all variables depending on the two HPLC columns used for the analysis

Factors	P-value (InertSustain Phenyl)	P-value (Synergi Polar)
	Linear effect	
Time	0.073	0.175
C_{EtOH}	0.098	0.513
pH	0.121	0.047*
	Quadratic effect	
Time*Time	0.035*	0.049*
C_{EtOH}*C_{ETOH}	0.384	0.796
pH*pH	0.019*	0.046*
	Interaction effect	
Time*C_{EtOH}	0.010*	0.009*
Time*pH	0.137	0.261
C_{EtOH}*pH	0.146	0.244

**Significant at p<0.05*

Analysis of the 5-CQA peak area using HPLC reveals significant interactions among extraction time, pH, and ethanol concentration. Contour plots show that prolonged extraction times and higher pH (above 6) increase 5-CQA concentrations, independent of the HPLC column used. This effect may relate to reversible isomerization of hydrolyzed 5-CQA products, which mainly involves acyl groups, at elevated pH levels and varying extraction conditions (Mok et al., 2022). The study indicates that shorter extraction times yield higher 5-CQA concentrations than prolonged heating across all tested ethanol concentrations and pH levels. Optimal

extraction parameters were determined to be 25% ethanol at pH 7 with a 20-minute heat treatment, yielding composite desirability values of 0.9455 and 0.9498 for Phenyl and Polar columns, respectively. The experimental values resulted in 5-CQA yields of 1.43 mg and 1.48 mg from 1 g of dried hibiscus flowers for Phenyl and Polar columns, respectively. In contrast to earlier findings reporting lower yields with methanol, this study concludes that ethanol is a more effective solvent for 5-CQA extraction, owing to its lower polarity. For example, Mok et al (2019) extracted a maximum of 0.12 mg/g of CGA from dry hibiscus flower using 50% methanol for 6 hrs. This finding shows that methods that reduce water's polarity increase the solubility of 5-CQA, a moderately polar compound, by releasing hydrogen ions. Ethanol, which has a larger alkyl group than methanol, is less polar and proves to be an ideal solvent for 5-CQA extraction (Mok et al., 2022). Previous research indicates that heat treatment significantly affects 5-CQA extraction; specifically, its acyl group on the carbon-5 hydroxyl of quinic acid makes it sensitive to heat. Extraction temperatures exceeding 110˚C and prolonged extraction times decrease 5-CQA yield (Mok et al., 2022). In contrast, the method introduced in this study is more efficient and sustainable in terms of solvent use and energy consumption during heat treatment for 5-CQA extraction.

4.3 Analysis of phenolics and caffeine concentration in natural deep eutectic solvent extracts of green coffee beans

4.3.1 Total phenolics in green coffee bean extracts

The solubility of phenolic compounds mainly depends on their chemical structure, particularly on the balance between polar hydroxyl groups and the non-polar aromatic ring. Chemical modifications such as esterification or glycosylation can influence the solubility of phenolics (Taheri &

Jafari, 2019). In addition, factors such as temperature, extraction time, pH, and the presence of salts or cosolutes also affect their solubility (Furia et al., 2021; Chandimala et al., 2025). TPC values detected in water, 50% ethanol, and a Glu-Glyc-based NADES system are shown in Figure 10.

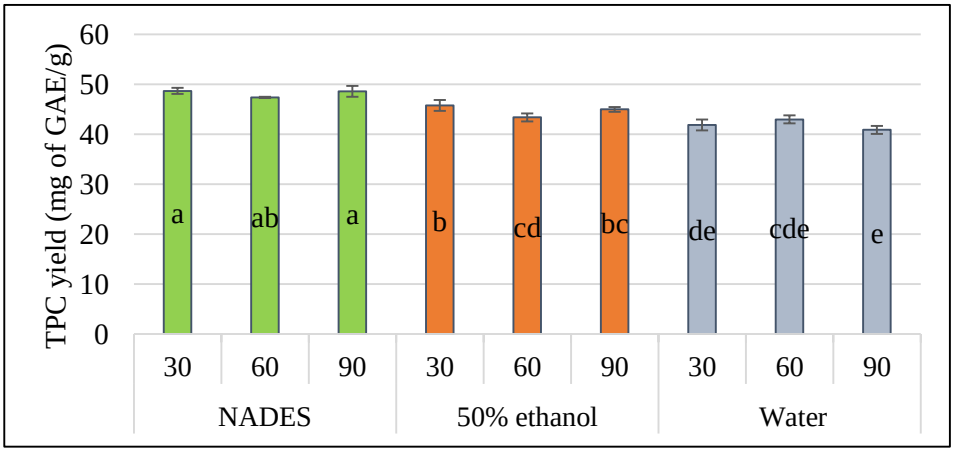


Figure 10 TPC of green coffee bean extracts with Glu-Glyc-based NADES, 50% ethanol, and water at 30˚C temperature at three different extraction times: 30, 60, and 90 min

Notes: Columns that depict the same letter are not significantly different at a 95 % confidence level according to Tukey's HSD test. Letters are assigned from a to e in descending order of the mean values. Error bars represent the SD of the mean.

The highest TPC yield (48.7±0.6 mg GAE/g) was observed in the NADES extract at 30-min extraction time, closely followed by the 90 min extract (48.6±0.6 mg GAE/g), with no significant difference between extraction times ($p>0.05$). Moreover, NADES produced significantly higher TPC at the 30 and 90 min extraction times than 50% ethanol and water ($p<0.05$). These results indicate that Glu-Glyc-based NADESs are effective alternatives to water and organic solvents such as ethanol for extract-

ing phenolic compounds from green coffee beans. The observed TPC content of 4.0-4.8% is comparable to previously reported values (3.8%) for water extracts of green coffee beans (Priftis et al., 2015). Previous studies using COSMO-SAC modelling have shown that phenolic compounds, such as CGAs, exhibit limited regions available for hydrogen bonding. In contrast, NADES mixtures have been reported to provide extensive hydrogen-bonding interaction sites and solvent networks compared to conventional solvents such as alcohols (Hijo et al., 2022). The higher TPC yields observed in NADES compared to other solvents may therefore be associated with their enhanced hydrogen-bonding capacity and favorable solute–solvent electrostatic interactions. These interactions have been proposed as contributing factors to the improved extraction efficiency of phenolic compounds in NADES systems. (Hijo et al., 2022). Sik et al. (2024) also found that Glu-Glyc-based NADES is more effective than conventional organic solvents (80% (v/v) ethanol) in extracting phenolic compounds from cornelian cherry. The current study indicated that the highest TPC yield at 30˚C for 30 min extraction time was statistically similar to that at 50˚C for 30 min extraction ($p>0.05$). These results suggest that a short extraction time at room temperature (~30˚C) is the most suitable for isolating phenolics from green coffee beans using Glu-Glyc-based NADES.

Additionally, optimizing the preparation of NADES is critical for improving the extraction efficiency of phenolic compounds. The hydrogen bond donor-to-acceptor ratio and water addition significantly affect the solvent's polarity and hydrogen-bonding capacity. Specifically, adding water enhances mass transfer, reduces viscosity, and increases polarity, thereby elevating phenolic yields (Hijo et al., 2022; Loukri et al., 2022). In the present study, pre-trials with Glu-Glyc mixtures indicated that a 5 mL

volume at a 5-fold dilution yields a higher TPC. For example, NADES extraction performed at 5 mL volume with a 10-fold dilution yielded lower TPC values of 39.9±1.9 and 43.4±2.1 at 30 and 60 min extraction times, respectively.

4.3.2 Chlorogenic acid concentration in green coffee bean extracts

Findings revealed that the highest yield of 5-CQA (41.5±0.5 mg/g) occurred at 30˚C through ethanolic extraction for 30 min, which was statistically similar ($p>0.05$) to that of all ethanolic extracts and NADES extracts for 60 and 90 min. However, NADES extraction for 30 min yielded 38.9±0.9 mg/g of 5-CQA, significantly lower ($p<0.05$) than the yield from the ethanolic extractions. Water extracts yielded the lowest 5-CQA concentrations, ranging from 34.3 to 34.8 mg/g. Similar results were observed for 4-CQA, with the highest yield coming from ethanolic extraction for 30 min (6.2±0.1 mg/g), comparable to NADES extractions for longer durations, while water extracts delivered lower levels. In terms of 3-CQA, no significant differences ($p>0.05$) were found between ethanolic and NADES extracts, but water extracts showed a significantly lower yield. Overall, the total CGA concentration was highest and similar all ethanolic extracts and NADES for longer extraction times. Water extracts again had the lowest yields of total CGA (43.9-44.4 mg/g).

The findings align with previous studies indicating that CGA comprises 3% to 6% of dry matter in *C. arabica* (Farah & Donangelo, 2006), confirming 5-CQA as the predominant phenolic compound across all types of extracts studied. In most cases, extending the extraction time from 30 to 90 min resulted in only marginal increases, suggesting that the majority of CGA is extracted within the first 30 min. However, the CGA yields in Glu-Glyc-based NADES were significantly lower than those of ethanol extracts

at certain extraction conditions (at 30˚C and 50˚C for 30 min). Being a polar molecule, CGA shows more affinity to polar solvents. Even with 50% water added to the Glu-Glyc, the 50% ethanol still can perform better because of the fundamental differences in how alcohol- and sugar-based solvents interact with CGA (Martins et al., 2025). Being an organic solvent, the chemical affinity provided by the aqueous ethanol can be more favorable for CGA with an aromatic ring. In addition, the strong hydrogen bond network in the NADES mixture (Hijo et al., 2022) can limit its capacity to accept more CGA molecules into the system.

4.3.3 Caffeine concentration in green coffee bean extracts

At 30˚C, ethanolic extracts showed comparatively higher caffeine yields than other extracts. The highest caffeine content (12.1±0.2 mg/g) was observed in 50% ethanol at 30˚C for a 30-min extraction. Findings indicated that there was no significant difference ($p>0.05$) in caffeine yield between all water and NADES extracts. Loukri et al. (2022) reported higher caffeine yields from coffee pulp using choline chloride-glycerin-based NADES solvent systems compared with the aqueous ethanolic system. In contrast, in the present study, ethanolic extraction for 30 min yielded significantly higher caffeine than all NADES extracts, which can be due to the higher polarity of 50% ethanol than the specific NADES composition used in this study. However, current findings suggest that Glu-Glyc-based NADES performs similarly to aqueous ethanol when longer extraction times are used at 30˚C for the extraction of caffeine from green coffee beans.

A detailed presentation of CGAs and caffeine concentrations in green coffee bean extracts are presented in Table 7.

Table7 CGA and caffeine concentrations in NADES, ethanol and water extracts of green coffee beans

Solvent	Extraction Temperature(˚C)	Extraction Time (min)	5-CQA (mg/g)	3-CQA (mg/g)	4-CQA (mg/g)	Total CGA (mg/g)	Caffeine (mg/g)
NADES	30	30	38.9±0.9^{b}	5.0±0.1^{a}	5.8±0.2^{b}	49.7±0.5^{b}	11.3±0.3^{b}
(Glu-Glyc)		60	39.2±0.6^{ab}	5.0±0.1^{a}	5.9±0.2^{ab}	50.1±0.3^{ab}	11.4±0.2^{ab}
		90	39.8±0.4^{ab}	5.0±0.1^{a}	6.0±0.1^{ab}	50.8±0.3^{ab}	11.5±0.2^{ab}
	50	30	38.1±0.6^{b}	4.8±0.1^{a}	5.7±0.1^{b}	48.6±0.3^{b}	11.1±0.2^{b}
50%	30	30	41.0±0.5^{a}	5.2±0.1^{a}	6.2±0.1^{a}	52.4±0.3^{a}	12.1±0.2^{a}
ethanol		60	40.1±0.6^{ab}	5.1±0.1^{a}	6.0±0.1^{ab}	51.1±0.4^{ab}	11.8±0.2^{ab}
		90	40.8±0.6^{ab}	5.2±0.1^{a}	6.1±0.1^{a}	52.0±0.3^{ab}	11.9±0.2^{ab}
	50	30	41.5±0.9^{a}	5.2±0.1^{a}	6.2±0.1^{a}	52.9±0.5^{a}	11.9±0.2^{ab}
Water	30	30	34.8±1.1^{c}	4.4±0.1^{b}	5.2±0.2^{c}	44.4±0.7^{c}	11.6±0.4^{ab}
		60	34.4±0.6^{c}	4.4±0.1^{b}	5.2±0.1^{c}	43.9±0.3^{c}	11.6±0.2^{ab}
		90	34.3±0.7^{c}	4.4±0.1^{b}	5.2±0.1^{c}	43.9±0.4^{c}	11.5±0.2^{ab}
	50	30	36.2±0.2^{bc}	4.6±0.1^{ab}	5.5±1.0^{c}	46.3±0.5^{bc}	11.3±0.3^{b}

Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different at a 95% confidence level according to Tukey's HSD test

4.3.4 Effect of extraction temperature on total phenolics, chlorogenic acid and caffeine concentrations of green coffee bean extracts

Figure 11 indicates that there was no significant difference ($p>0.05$) in TPC yields at 50˚C compared to 30˚C in all the tested extracts. The total CGA yield had no change in NADES or ethanol extracts at the higher temperature but significantly increased in water extracts. Notably, total CGA concentrations surpassed TPC in all green coffee bean extracts, particularly highlighting a greater disparity in ethanolic extracts. This can be because TPC measurements are not specific to all phenolic compounds, and include a broad range of phenolics, which may be degraded during the extraction. Additionally, the extraction efficiency for different phenolic compounds may vary with the method and conditions used in the extraction (Hijo et al., 2022). Caffeine yields at elevated temperature showed no significant difference ($p>0.05$) with those at 30˚C in all extracts.

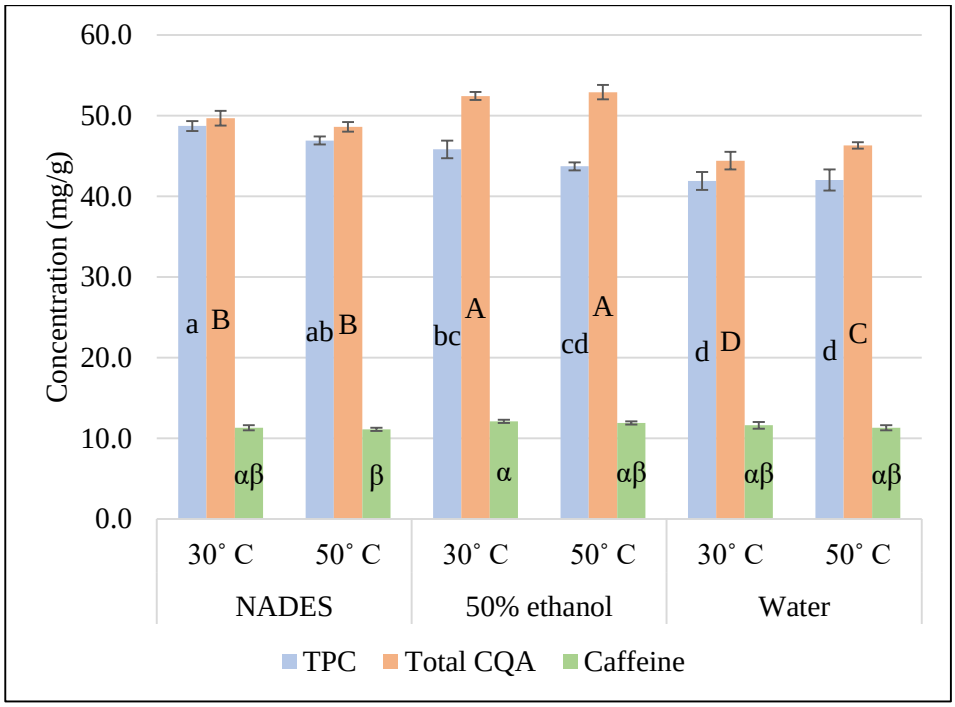


Figure 11 The effect of temperature increase (from 30˚C to 50˚C) on TPC, total CQA, and caffeine content of green coffee bean extracts (30 min extraction time, solvents—Glu-Gly-based NADES, 50% ethanol and water)

Notes: Values denote the mean of the three replicates based on independent one-way ANOVA conducted separately for each compound. Means followed by the same letter are not significantly different ($p>0.05$) at a 95 % confidence level according to Tukey's HSD test. Error bars represent the SD of the mean.

4.4 Phenolic compounds in natural deep eutectic solvent extracts of roselle calyx

4.4.1 Total phenolic content in roselle calyx extracts

At 30˚C, Glu-LA exhibited the highest TPC of 19.25±0.34 mg GAE/g, closely followed by Glu-CA at 18.61±0.15 mg GAE/g, which was statistically similar ($p>0.05$). The two ethanolic extracts (80% ethanolic extract=16.33±0.24 mg GAE/g; acidified 80% ethanolic extract=16.04±0.21 mg GAE/g) showed significantly lower yields ($p<0.05$), while the lowest TPC was observed in the Glu-Glyc extract (15.2±0.45 mg GAE/g). At 50˚C, TPC ranged from 17.69±1.07 mg GAE/g to 22.96±1.06 mg GAE/g, with Glu-LA showing the highest value, significantly higher than all the other extracts ($p<0.05$), indicating significant solvent-dependent differences in TPC yields (Figure 12). This result aligns with findings by Alañón et al. (2020), who reported comparable TPC using an oxalic acid and potassium chloride-based NADES extraction. Furthermore, current study demonstrates comparatively higher extraction efficiency than Kebede et al. (2025), who reported a maximum TPC of 6.73 mg GAE/g using an 80% methanol, and Manjula et al. (2022), who found a maximum of 12.84 mg GAE/g with an acidified 85% ethanol. This may be attributed to Glu-LA's unique polarity and hydrogen-bonding properties, which can enhance interactions between phenolics, such as protocatechuic acid and hibiscus acid, facilitating more effective extraction (Kebede et al., 2025).

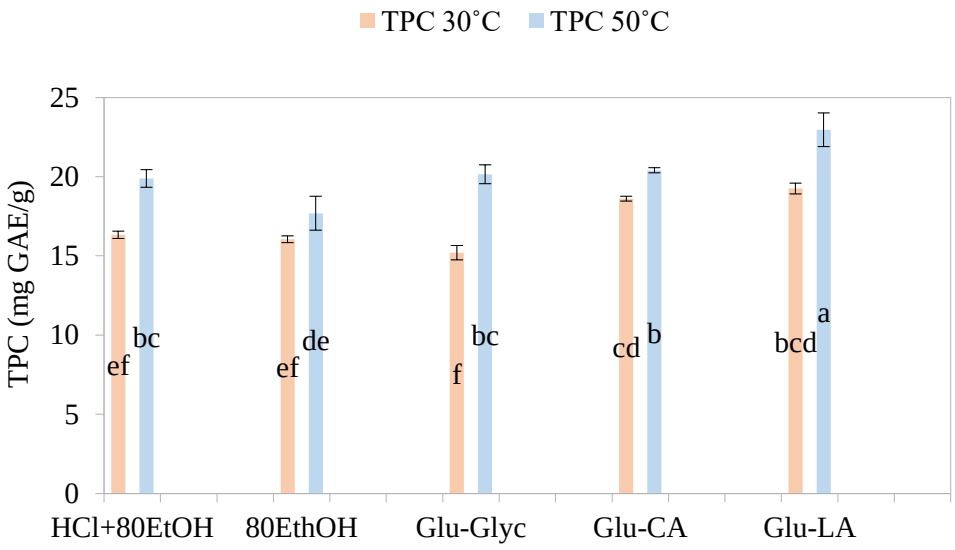


Figure 12 TPC yields (mg GAE/g) of roselle calyx extracts of NADES—Glu-Gly, Glu-CA, and Glu-LA, 0.1% HCl+80% (v/v) ethanol, and 80% (v/v) ethanol

Notes: Different letters denote significant differences ($p<0.05$) at a 95 % confidence level according to Tukey's HSD test. Error bars represent the SD of the mean.

Organic acid-based NADESs showed higher phenolic yields, likely due to their ability to disrupt plant cell walls, which enhances solvent-phenolic interactions (Toleugazykyzy et al., 2025). In contrast, the Glu-Glyc mixture was less effective as it lacks organic acids, resulting in lower phenolic outputs. Furthermore, an increase in extraction temperature from 30˚C to 50˚C notably improved TPC yields in Glu-Glyc due to reduced viscosity and enhanced extraction efficiency (Wang et al., 2024a) as the

elevated temperature appears to weaken cell walls, making extraction more accessible for solvents (Singh et al., 2021).

4.4.2 Total flavonoid content in roselle calyx extracts

At 30˚C, the highest TFC was observed in the 80% ethanol extract, measuring 2.92±0.08 mg QE/g, and was comparable to all the other extracts ($p>0.05$). At 50˚C, TFC ranged from 2.60±0.06 mg QE/g to 3.34±0.02 mg QE/g, with the highest TFC reported in ethanolic extracts (Figure 13).

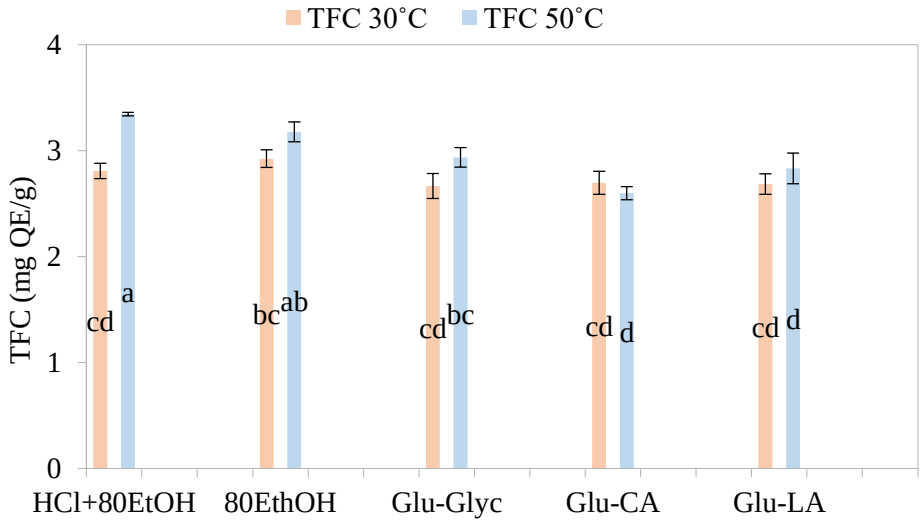


Figure 13 TFC yields (mg QE/g) of roselle calyx extracts NADES—Glu-Gly, Glu-CA, and Glu-LA, 0.1% HCl+80% (v/v) ethanol, and 80% (v/v) ethanol

Notes: Different letters denote significant differences ($p<0.05$) at a 95% confidence level, according to Tukey's HSD test. Error bars represent the SD of the mean.

Comparatively higher performance of ethanolic extracts over NADES at both temperatures may be associated with ethanol's intermediate polarity, which enhances the solubility of methoxylated flavonoids such as hibiscetin via dipole-dipole interactions and limits competition for flavonoid hydroxyl groups (Kebede et al., 2025). However, these results were contrary to those of Sallustio et al. (2024), who reported lactic acid and sodium acetate-based NADES being more effective than hydroalcoholic mixtures in extracting flavonoids from blackthorn and rosehip pulps.

Only the acidified ethanolic extract exhibited a significant increase ($p<0.05$) in TFC with rising temperature, likely due to enhanced diffusion and solubility of flavonoids at 50˚C. Zhu et al. (2025) reported a similar increase in TFC in ethanolic propolis extracts with increased temperature. Aguilar et al. (2023) and Wang et al. (2024a) reported that sugar-alcohol-based NADES demonstrated a strong propensity to form hydrogen bonds with flavonoids, effectively preventing their self-aggregation at 50˚C. However, in this study Glu-Glyc showed similar TFC yields at both temperatures. Furthermore, the temperature increase had a negligible impact on TFC yields from organic acid-based NADES, likely due to the disruption of hydrogen bonds at higher temperatures, which reduces surface tension and hampers the solvent's ability to disperse flavonoid aggregates (Aguilar et al., 2023). Overall, our findings suggest that NADESs should be further optimized to provide a more favorable environment for extracting polar compounds like flavonoids that contain more hydroxyl groups. Moreover, the TFC yields observed were lower than those reported by

Nerdy et al. (2022) but higher than those reported by Kebede et al. (2025), underscoring the significant influence of extraction conditions and plant origin on the results.

4.4.3 Total monomeric anthocyanin content in roselle calyx extracts

TMA content focuses on naturally occurring monomeric anthocyanins while excluding degraded polymeric forms that lack color-changing properties and health benefits (Lee et al, 2005). The predominant anthocyanins identified in roselle calyx are delphinidin-3-sambubioside and cyanidin-3-sambubioside, which, along with their derivatives, impart the calyx its characteristic red hue and contribute to its biological activities (Cira-Chávez et al., 2025). Research indicates that the composition of *H. sabdariffa* L. calyx features these anthocyanins in an 80:20 ratio (Cira-Chávez et al., 2025).

At 30˚C, the Glu-LA extract exhibited the highest TMA content, with a value of 8.42±0.05 mg DSE/g, closely followed by the Glu-CA extract at 8.02±0.26 mg DSE/g, showing statistical similarity ($p>0.05$). Lower TMA yields were recorded for the 80% ethanolic extract (7.29±0.23 mg DSE/g) and the acidified ethanolic extract (7.03±0.23 mg DSE/g), which were also statistically comparable. The lowest TMA yield was observed in the Glu-Glyc extract at 5.58±0.12 mg DSE/g. At 50˚C, TMA content varied among extracts, from 7.50±0.17 mg DSE/g in acidified ethanolic extract to 8.13±0.43 mg DSE/g in Glu-LA, but no significant differences were found among all extracts ($p>0.05$) at this temperature (Figure 14).

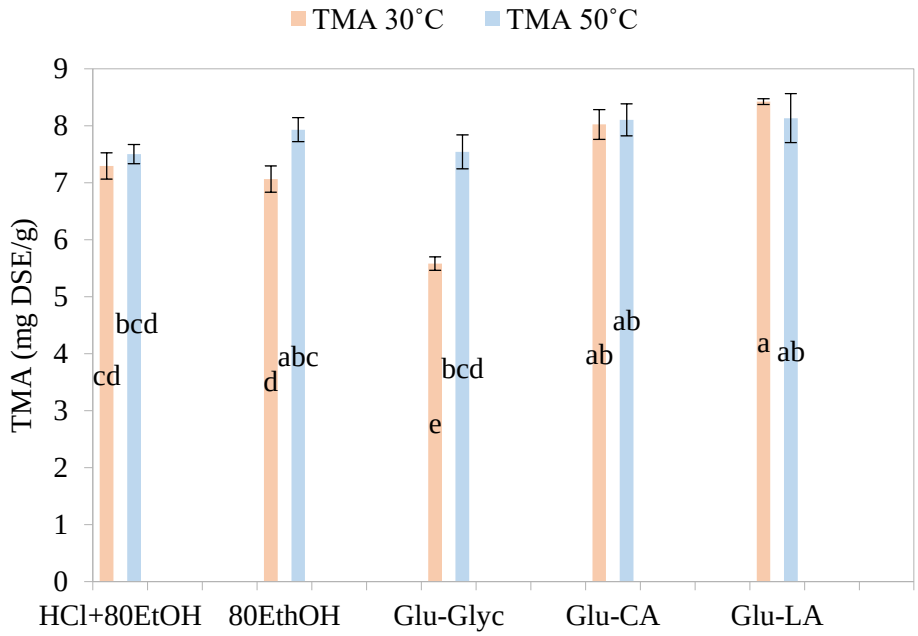


Figure 14 TMA content (mg DSE/g) of roselle calyx extracts NADES—Glu-Gly, Glu-CA, and Glu-LA, 0.1% HCl+80% (v/v) ethanol, and 80% (v/v) ethanol

Notes: Different letters at the same temperature extracts denote significant differences ($p<0.05$) at a 95 % confidence level according to Tukey's HSD test. Error bars represent the SD of the mean.

The study concluded that organic acid-based NADES extracts, especially Glu-LA, yielded higher TMA contents than sugar-alcohol NADES, as supported by prior research (Dai et al., 2016; Grillo et al., 2020). The higher polarity of organic acid-based NADES was noted to facilitate the solubilization and stabilization of anthocyanins, which thrive

in acidic conditions (Dai et al., 2016). However, studies report that citric acid-based NADES show lower extraction efficiency due to steric hindrance from its numerous carboxyl and hydroxyl groups (Grillo et al., 2020). The lower viscosity of organic acid-based NADESs was also recognized as a factor influencing anthocyanin mass transfer (Zannou et al., 2024), with Glu-Glyc's high viscosity significantly impacting TMA yield, as evidenced by increased yields at elevated temperatures with lower viscosity (Wang et al., 2024b).

4.4.4 Antioxidant activity of roselle calyx extracts

The study evaluates the antioxidant activity of hibiscus extracts using the DPPH method, which measures their ability to scavenge free radicals. It was found that extracts rich in phenolic compounds exhibit higher activity, with notable differences in activity depending on solvent and temperature (Figure 15). At 30˚C, the highest activity was observed in Glu-LA (14.5±0.3 mg AAE/g), followed by Glu-CA and the ethanolic extracts. Conversely, Glu-Glyc exhibited the lowest activity (10.8±0.5 mg AAE/g). At 50˚C, Glu-LA maintained the highest activity (13.9±0.8 mg AAE/g), which was significantly greater ($p<0.05$) than the acidified ethanolic extract with the lowest value (11.2±0.9 mg AAE/g). It was noted that extracts with higher TPC and TMA generally exhibit greater antioxidant capacity,

although the contributions can vary depending on the solvent and extraction technique (Anggraini et al., 2019).

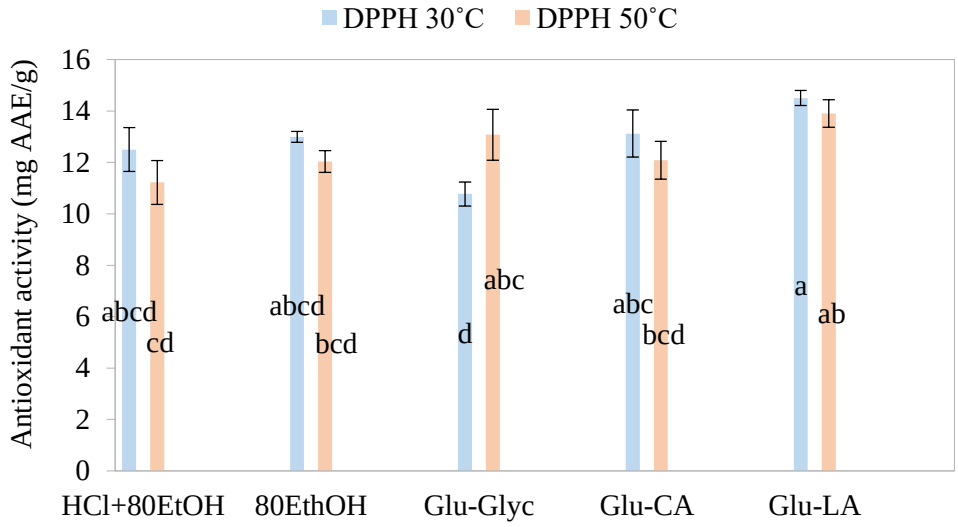


Figure 15 DPPH radical scavenging activity (mg AAE/g) of roselle calyx extracts NADES—Glu-Gly, Glu-CA, and Glu-LA, 0.1% HCl+80% (v/v) ethanol, and 80% (v/v) ethanol

Notes: Different letters denote significant differences ($p<0.05$) at a 95% confidence level according to Tukey's HSD test. Error bars represent the SD of the mean.

Moreover, recent findings indicate that NADESs can enhance antioxidant activity compared to conventional organic solvents, which may be due to the effects of certain NADES components that act as radical absorbers (Foroutani et al., 2024), making antioxidant activity measurements of NADES extracts challenging. The superior efficacy of Glu-LA at both

temperatures suggests its optimal polarity for targeting phenolics with antioxidant characteristics, recommending its use in applications focused on alleviating oxidative stress, such as food formulations.

4.4.5 Chlorogenic acid content in roselle calyx extracts

Table 8 shows the concentration of CGA isomers (3-CQA, 5-CQA, and 4-CQA) found at two extraction temperatures (30˚C and 50˚C) in ethanolic and NADES extracts, based on HPLC measurements using an InertSustain Phenyl column. As shown in Table 8, 3-CQA was the most prominent CGA (~52% w/w) in roselle calyx extract. Total CGA concentration at 30˚C was highest in the 80% ethanolic (2.65±0.02 mg/g) extract, while the lowest was in the Glu-Glyc (1.89±0.03 mg/g) extract. At 50˚C, total CGA concentrations ranged from 2.44±0.04 mg/g to 2.61±0.02 mg/g across the investigated extracts.

The effects of temperature on TPC, TFC, TMA, DPPH radical scavenging activity, and CGA yield are summarized in Table 9. It is shown that increasing the extraction temperature has a significant effect ($p<0.05$) on TPC (+19.3%), while it has a negligible impact ($p>0.05$) on other components: TFC (+5.6%), TMA (-6.9%), DPPH activity (-3.5%), and CGA concentration (-1.6%).

Table 8 CGA concentration (mg/g) of roselle calyx extracts

Extraction temperature (℃)	Solvent	3-CQA	5-CQA	4-CQA	Total CGA
30	0.1% HCl+80%EthOH	1.38±0.01^{ab}	0.56±0.01^{ab}	0.63±0.02^{a}	2.57±0.02^{b}
	80%EthOH	1.41±0.01^{a}	0.59±0.02^{a}	0.65±0.01^{a}	2.65±0.02^{a}
	Glu-Glyc	1.03±0.02^{d}	0.40±0.02^{c}	0.46±0.02^{c}	1.89±0.03^{d}
	Glu-CA	1.32±0.02^{c}	0.55±0.00^{b}	0.62±0.03^{ab}	2.48±0.03^{bc}
	Glu-LA	1.34±0.02^{bc}	0.58±0.02^{ab}	0.62±0.00^{ab}	2.54±0.03^{bc}
50	0.1% HCl+80%EthOH	1.40±0.02^{a}	0.59±0.01^{a}	0.63±0.01^{a}	2.61±0.03^{ab}
	80%EthOH	1.40±0.02^{a}	0.57±0.02^{ab}	0.64±0.00^{a}	2.61±0.02^{ab}
	Glu-Glyc	1.31±0.04^{c}	0.54±0.01^{ab}	0.59±0.01^{b}	2.44±0.04^{c}
	Glu-CA	1.32±0.01^{c}	0.56±0.01^{ab}	0.62±0.02^{ab}	2.50±0.02^{bc}
	Glu-LA	1.32±0.01^{c}	0.57±0.01^{ab}	0.61±0.02^{ab}	2.50±0.02^{bc}

Notes: Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different (p>0.05) at a 95% confidence level according to Tukey's HSD test

Table 9 Effect of temperature on the phenolic yields of roselle calyx extracted with Glu-LA-based NADES

Extraction temperature (℃)	TPC (mg GAE/g)	TFC (mg QE/g)	TMA (mg DSE/g)	DPPH radical scavenging activity (mg AAE/g)	Total CGA concentration (mg/g)
30	19.25±0.34[b]	2.68±0.10[a]	8.42±0.05[a]	14.50±0.30[a]	2.54±0.03[a]
50	22.96±1.06[a]	2.83±0.15[a]	8.13±0.43[a]	13.90±0.80[a]	2.50±0.02[a]

Notes: Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different ($p>0.05$) at a 95% confidence level according to Tukey's HSD test

In this study, ethanolic extracts, which are moderately polar substances, proved more effective at extracting CGA at 30˚C. However, at 50˚C, Glu-LA and Glu-CA resulted in CGA yields statistically similar to ethanolic extracts. In a study, Fan et al. (2025) reported that betaine-glycerol-based NADES extracts contained 1.9 times more CGA than ethanolic extracts of *Lonicera japonica*. The current results may be due to the specific NADES mixtures and their compositions used in this study, and the effect of temperature on polarity. Further adjustments to optimize molar ratios of organic acid-based NADES could possibly enhance CGA yields by increasing regions suitable for hydrogen bonding and interactions (Hijo et al., 2022). The lower extraction efficiency observed with Glu-Glyc-based NADES may be attributed to hindered solvent movement and the slower diffusion of the extracted CGA, both resulting from its higher viscosity (Zannou et al., 2024). Literature reports indicate that Glu-CA- and Glu-LA-based NADES containing 10%-30% water by mass have viscosities of 37.9 mPa.s and 46.24 mPa.s, respectively. Savi et al. (2019) reported that Glu-LA NADES containing 30-50% water exhibits an apparent viscosity of 5 mPa.s at 90˚C and 1.27 g/mL of density and Newtonian flow behavior at room temperature. This density was close to the density measurement (1.15 g/mL) recorded in our experiment. According to Savi et al. (2019) surface tension of a Glu-LA mixture without water measures 37.42 mN/m, decreasing to 35.01 mN/m with the addition of 30-50% water, which were considerably lower than those of choline chloride-based NADES. Glycerol-based NADESs, however, have

significantly higher viscosities. For instance, glycerol-fructose-based NADES exhibits a viscosity of 383.9 mPa.s (Koh et al., 2024). Therefore, these results suggest that the molar ratio and water content in Glu-Glyc-based NADES should be optimized to reduce viscosity and improve the extraction of phenolics, including CGA. Generally, these results emphasize that specific properties of NADES (e.g., viscosity, polarity, and hydrogen bonding capacity) are imperative factors to consider when extracting bioactive compounds from plant extracts. Overall, Glu-LA- and Glu-CA-based NADES remain competitive with ethanolic extractions for CGA extraction at elevated temperatures. Therefore, choosing the solvent or formulation of the NADES and temperature should be performed considering the target bioactive compound of interest.

Considering the potential limitations, such as volatility, flammability, and toxicity of organic solvents (e.g., ethanol, methanol) commonly used for extracting phenolics from roselle calyx, it is crucial to explore non-conventional, innovative green solvents for isolating phenolics from roselle calyx (Foroutani et al., 2024). Overall, obtained results indicates that organic acid-based NADES (Glu-LA and Glu-CA) yield higher amounts of phenolics from roselle calyx and are promising green solvents. Among them, Glu-LA-based NADES outperformed other NADES in nearly all tests under the given extraction conditions. Therefore, Glu-LA is recommended as the most efficient NADES for extracting phenolics from roselle calyx. Previous studies also suggest that organic acid-based NADES are effective for extracting bioactive compounds from plants,

with improved efficiency and stability (Socas-Rodríguez et al., 2021; Zannou et al., 2024). This is mainly because acidic NADES act as multifunctional hydrogen bond donors, forming strong cross-interactions in the mixtures (Zannou et al., 2024). However, it is important to find the right balance between phenolic extraction efficiency and their stability within a specific NADES. For example, interactions between NADES and flavonoids can increase flavonoid solubility, but strong bonds might also make it difficult to break these interactions, reducing overall extraction efficiency (Wang et al., 2024a). Since acidic NADES offer tunable polarity through altered molar ratios (Zannou et al., 2024), optimization can be easily performed to match the polarity of phenolics in the roselle calyx. Considering the overall results of this experiment, Glu-LA NADES at 50˚C for a 30-min extraction was selected to continue the ENC-related experiments.

4.5 Evaluation of encapsulated natural deep eutectic solvent extracts of roselle calyx

Based on the results described in Section 4.4, a Glu-LA NADES mixture was utilized for extracting phenolics from roselle calyx for further ENC purposes (Figure 16). The density of Glu-LA-based roselle extract used for the ENC was 1.15±0.01 g/mL.

Figure 16 (Left) Glu-LA extract of roselle calyx (30˚C for 30 min extraction); (Right) freeze-dryer used for further ENC of roselle extract

4.5.1 Encapsulation efficiency, drying yield, moisture content, visual characteristics and functional properties of encapsulated powder

After mixing coating material blends with roselle extracts, the samples containing only GA and a higher amount of GA appeared more viscous, resulting in a slightly soggy texture after ENC due to poor ice crystal formation during freezing (Figures 17). This finding was also supported by Fitri et al. (2020). Drying yield, moisture content, visual characteristics (as observed with naked eye) (Figure 18) and ENC efficiency of different combinations of MD and GA as wall material are given in the Tables 10 and 11.

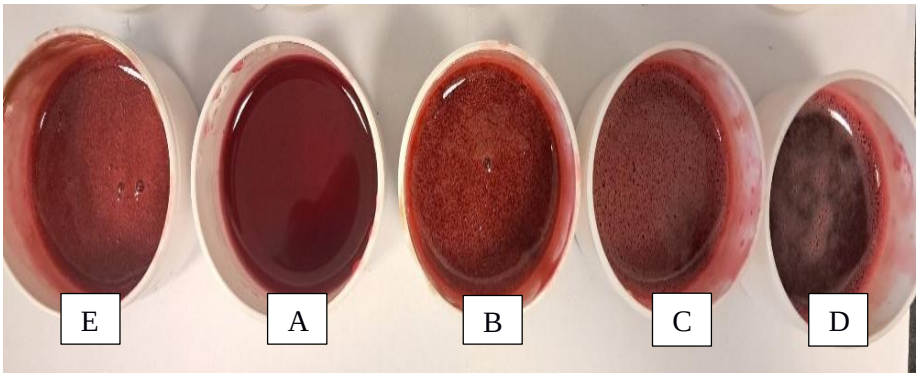


Figure 17 Wall material mixtures used for the ENC (MD-to-GA ratios of 1:0 (A), 1:3 (B), 1:1 (C), 3:1 (D), and 0:1 (E))combined with roselle calyx extracts before ENC

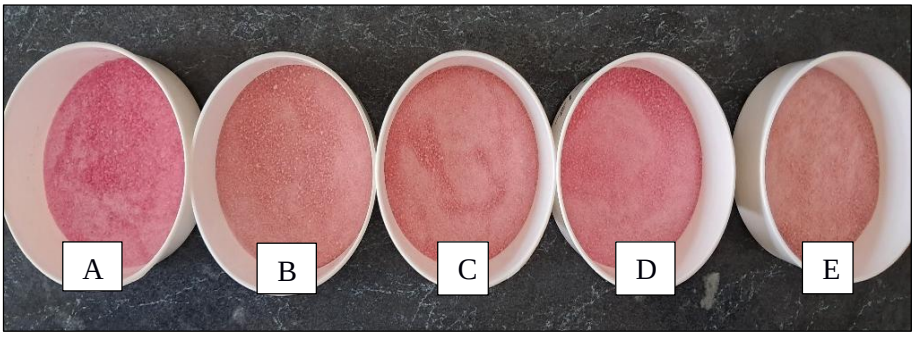


Figure 18 Glu-LA extracts of roselle calyx after freeze-drying ENC with different wall material mixtures (MD-to-GA ratios of 1:0 (A), 1:3 (B), 1:1 (C), 3:1 (D), and 0:1 (E))

Table 10 Drying yield, moisture content, and visual descriptions of freeze-dried encapsulated roselle

Treatment ID	MD:GA	Drying yield (%)	Moisture content (%)	Visual characteristics
A	1:0	86±0.01[a]	5.13±0.00[a]	Bright pink and non-sticky powder
B	1:3	89±0.01[a]	5.46±0.00[a]	Pink with a light orange hue, non-sticky powder
C	1:1	90±0.01[a]	5.41±0.00[a]	Pink with a dark orange hue, non-sticky powder
D	3:1	85±0.04[a]	5.31±0.00[a]	Pink with a light red hue, non-sticky powder
E	0:1	87±0.01[a]	5.86±0.01[a]	Pink with a light orange hue, slightly sticky powder

Notes: Values denote the mean±SE of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different ($p>0.05$) at a 95% confidence level according to Tukey's HSD test

Table 11 ENC efficiency of freeze-dried encapsulates of roselle

Treatment ID	MD:GA	Total anthocyanin (mg DSE/g)	Surface anthocyanin (mg DSE/g)	ENC Efficiency (%)
A	1:0	0.34±0.04[a]	0.04±0.01[a]	87±0.03[b]
B	1:3	0.25±0.03[a]	0.02±0.01[b]	92±0.05[ab]
C	1:1	0.34±0.05[a]	0.01±0.00[b]	97±0.01[ab]
D	3:1	0.36±0.03[a]	0.00±0.01[b]	99±0.02[a]
E	0:1	0.26±0.00[a]	0.00±0.00[b]	98±0.00[a]

Notes: Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different (p>0.05) at a 95% confidence level according to Tukey's HSD test

The drying yield of encapsulated powder is the ratio of the actual weight of dehydrated roselle powder resulting from the freeze-drying process to the total weight of the solid material (core material and wall material) fed to the dehydration system (Pudziuvelyte et al., 2020). Lyophilization typically generates higher drying yields, often 75% to 100%, indicating greater efficiency and less product loss during processing. This is one of the main reasons lyophilization is superior to spray-drying, as spray-drying causes losses due to sticking to the chamber walls. Nevertheless, the yield is highly dependent on the wall material composition, MD and GA mixtures typically showing higher powder recovery (above 78%) (Zhao et al., 2025). In the present study, no significant difference in drying yield between treatments was observed

($p>0.05$), although the highest yield (90±0.01%) was recorded in encapsulates with 1:1 MD-to-GA. Final products characteristically exhibited low moisture content, ranging between 5–6%, depicting enhanced stability. There was no significant difference in moisture content among treatments ($p>0.05$), with the highest value found in the sample containing only GA and the lowest in the sample with only MD, which can be due to the more hygroscopic nature of GA compared to MD. ENC efficiency measures the percentage of an active ingredient that is successfully entrapped within a carrier matrix or capsule relative to the total amount added during production. It characterizes the formulation's success and reflects drug-delivery efficacy. High ENC efficiency ensures high product quality, better therapeutic efficiency, lower costs, and less waste (Azarpazhooh et al., 2022). ENC efficiency was significantly lower in the treatment with only MD, likely because MD lacks emulsifying capacity and forms, hypothetically a porous, brittle film that allows volatile anthocyanins to escape. Overall, the combination of GA and MD resulted in improved ENC efficiency compared to MD alone, suggesting that GA contributes positively to matrix formation and stabilization. The observed improvements may be attributed to the complementary properties of the two wall materials, where GA provides strong film-forming and emulsifying characteristics, while MD contributes to structural stability (Cid-Ortega & Guerrero-Beltrán, 2020). These results indicate that combining GA with MD can create a synergistic, stable

matrix for roselle phenolic compounds, emphasizing the importance of balancing MD and GA to improve ENC efficiency.

Recent studies indicate that freeze-drying ENC of plant extracts effectively preserves bioactive components, resulting in higher retention rates, greater bioaccessibility, and improved storage stability (Fitri et al., 2020). Nevertheless, this technique poses significant challenges for commercial use, including porous structure in created powder, which makes the encapsulated powder fragile, prone to oxidation and moisture absorption, and lowers its shelf life and elevated energy consumption, extended processing times, and limited production capacity (Zhao et al., 2025). In the present study, boiling over of the samples during the primary dehydration stage of lyophilization was a serious problem, and it was addressed by carefully altering the wall-to-core material ratio during the preliminary trials. Organic acids such as lactic acid are particularly challenging to lyophilize, as they often remain as a syrup even at very low temperatures. Studies suggest that NADES mixtures comprising lactic acid (e.g., Glu: LA 1:5) exhibit glass transitions below -53˚C (Savi et al., 2019). After the preliminary trials, the wall-to-core material ratio was adjusted to 2.5:1 in all treatments and freezing was continued for at least 12 hours at -80˚C until the sample reached a solid glassy state before starting the vacuum, to address the boiling over problem.

Treatment D, with a 3:1 MD-to-GA ratio, was selected for further experiments because it demonstrated the highest ENC efficiency. The encapsulated powder showed a content of 2.50±0.08 mg GAE/g of TPC,

0.36±0.03 mg DSE/g of TMA, and 0.36±0.04 mg AAE/g of DPPH-radical scavenging activity. The TPC and TMA levels in the encapsulated powder are relatively low compared to those of the pure plant extract, which contains 22.96 mg GAE/g of TPC, 8.13 mg DSE/g of TMA and 13.90±0.80 mg AAE/g of DPPH-radical scavenging activity. This indicates that the powder is highly diluted with the excipients (MD and GA), a common practice for protecting unstable compounds, and demonstrates that phenolic compounds are satisfactorily encapsulated and stabilized. However, the nearly negligible TMA concentration in the encapsulated powder suggests that storage of the NADES extract before processing or during freeze-drying led to significant anthocyanin degradation in the roselle calyx, which represents a limitation of this study. Ćujić Nikolić et al. (2023) and Tarone et al. (2020) also reported a considerable decrease in anthocyanin contents in encapsulated powder after chemical analysis, although the ENC occurred due to physical incorporation. This can be affected by the wall material and the solvent used to extract phenolics from the encapsulated powder. For instance, Li et al. (2020) reported that 16% more total anthocyanins could be extracted from MD-based microencapsulates when acidified methanol was used for the extraction. Overall, the characteristics of wall material components, their chemical origin, and interactions within the complex, as well as with reagents used in tests, can affect the stabilization or release of phenolics, including anthocyanins in the encapsulated powder (Ćujić Nikolić et al., 2023). Additionally, the modest DPPH-radical scavenging activity of the

encapsulated powder underscores its antioxidant potential. These findings show that freeze-dried ENC satisfactorily preserved a stable, though low, concentration of phenolic compounds in the roselle calyx. Therefore, this powder could serve as a modest antioxidant ingredient, suitable for foods where high color intensity or high-dose phenolic supplementation is unnecessary.

4.5.2 Powder properties and pH stability of encapsulated powder

The selected encapsulated powder demonstrated a bulk density of 0.55±0.00 g/mL and a tapped density of 0.61±0.01 g/mL, resulting in a Carr index of 10%±0.00% and a Hausner ratio of 1.11±0.00. In pharmaceutical and food science, these parameters are considered standard indicators of the ease with which an encapsulated powder can be processed, packaged, and transported (Ćujić Nikolić et al., 2023). The bulk density value is moderate for a freeze-dried encapsulate, which is typically porous and lightweight. The small increase from bulk to tapped density indicates that the powder particles are already efficiently packed in their loose state, suggesting they may have a spherical or regular shape, which prevents the formation of large voids, preventing collapse during tapping (Schlick-Hasper et al., 2022). An excellent (<10%) Carr index value, which indicates “free-flow”, also reflects low interparticle cohesiveness and friction. A Hausner Ratio of 1.11 indicates "excellent" flow properties, indicating the powder's minimal compressibility under mechanical stress, characteristic of free-flowing materials (Schlick-Hasper et al., 2022). Overall, these powder properties exhibit excellent flowability

and low compressibility of the encapsulated powder, making it ideal for high-speed automated ENC, ensuring consistent dosage uniformity.

Additionally, the encapsulated powder exhibited a complete dissolution time of 51±1.14 s, indicating excellent reconstitution properties (Figure 19).

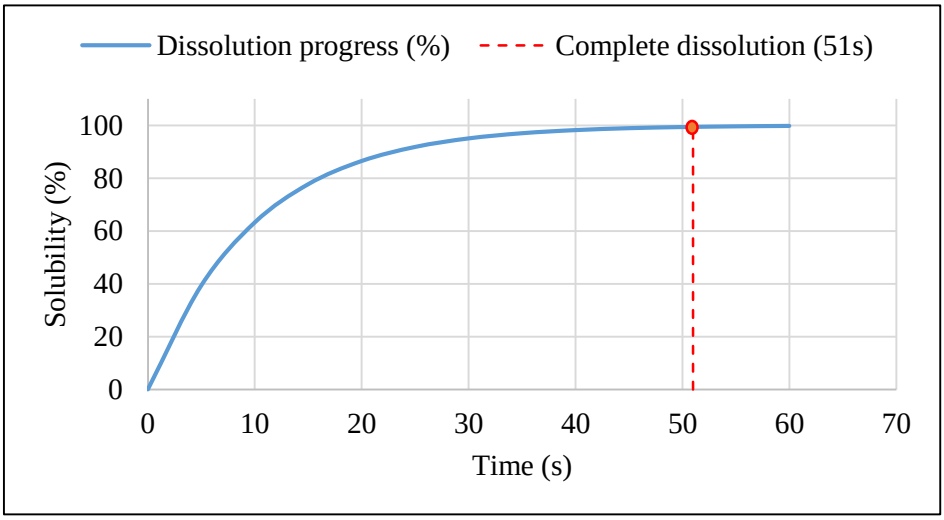


Figure 19 Time-dependent solubility of roselle freeze-drying encapsulated powder (with wall material composition of 3:1 MD-to-GA ratio) at 30±2˚C temperature

This may be due to the superior porous structure of lyophilized powders, which allows rapid water absorption via capillary action, while other factors, such as pore size distribution, may also play a significant role. Moreover, this indicates that the wall material components, MD and GA, are highly hydrophilic and have effectively shielded hydrophobic ingredients in the core materials. These properties suggest that the

encapsulated powder will be easy to mix without specialized high-shear equipment in an industrial or clinical setting. Furthermore, as the test was performed using water at room temperature, the results indicate that the powder exhibits the potential for its application as a premium instantized product ready for direct-consumer use, after broader application-specific validation.

According to Figure 20, a considerable instability can be observed at pH 6.8 during the first two hours, indicating a bathochromic shift (the color changing from red to blue/purple) or the formation of degradation products that absorb more strongly at this wavelength (Lee et el., 2005), followed by a decline in the value after 3 hours, suggesting the pigment has likely degraded or precipitated. At pH 4.5, the absorbance was lower, probably due to the pigment shifts toward the colorless hemiketal form (Lee et el., 2005). At pH 2.0, the highest initial absorbance is maintained in a relatively steady, though slightly declining profile. At pH 1.0, the observed irregular trend with a rise in absorbance at hour 3 suggests a potential delayed release of the extract from the ENC matrix over time (Lee et el., 2005). Overall, the pH stability test demonstrated that the encapsulated powder is highly sensitive to neutral conditions (pH 6.8) and should be utilized in acidic environments (pH 1.0–2.0) for maximum color retention. The data at pH 1.0 suggests that the ENC method might provide controlled release.

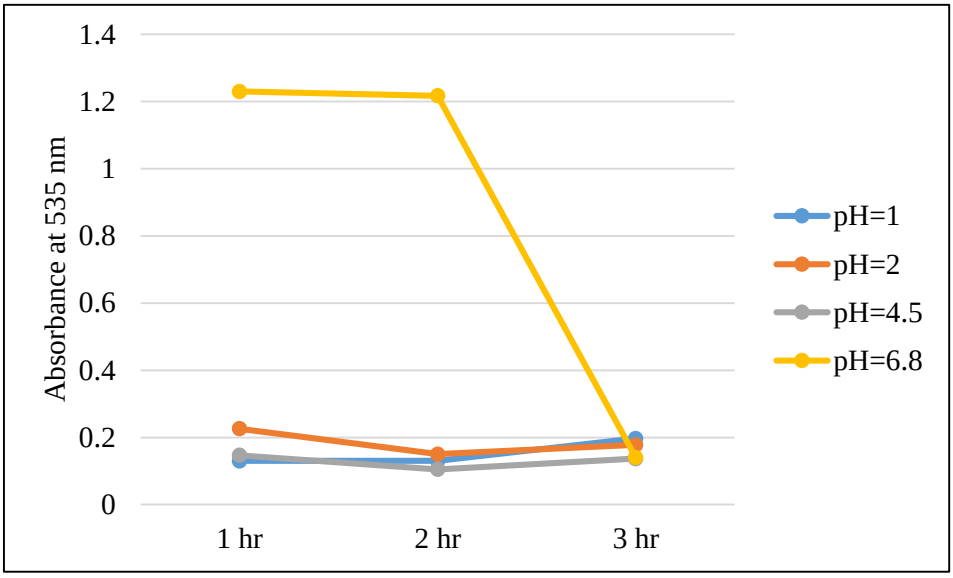


Figure 20 Spectrophotometric absorbance profile of roselle freeze-dried encapsulated powder (with MD-to-GA ratio of 3:1) at varying pH levels at 25±2˚C temperature

The degradation rate constant (*k*) calculated using Eq.9 for the encapsulated powder at pH 6.8 was 1.087/hr, confirming that the roselle calyx encapsulate is highly unstable at pH 6.8. Sinela et al. (2017) also confirmed that anthocyanins follow first-order kinetics and are highly unstable at neutral pH (above 6.0). On the contrary, at pH 1.0, a release profile with an initial lag phase was observed. This suggests that the wall material likely resisted hydration for the first 2 hours, but then began to dissolve or swell rapidly by the 3rd hour, releasing the anthocyanins. This highlights a desirable trait of preserving the color during early storage but releasing it later (e.g., during digestion or after a specific shelf-life period). Furthermore, data analysis using the Korsmeyer-Peppas model as described by

Korsmeyer et al. (1983), yielded a release exponent (n=1.02), indicating a tendency towards super-case II transport, where the release of anthocyanins is driven primarily by swelling and structural erosion of the wall material rather than by simple Fickian diffusion, as observed with this empirical model. However, to precisely determine the color stability of the encapsulated powder, extended absorbance measurements are required.

4.6 Characteristics of Túró Rudi with roselle encapsulated powder

4.6.1 Functional properties of Túró Rudi with roselle encapsulated powder

TPC, TMA, and antioxidant activity of Túró Rudi developed with roselle encapsulated powder are described in Table 12. According to the results, a significant increase in the TPC can be observed at the 9% fortification level (T3) owing to the natural richness of roselle calyx in polyphenols such as protocatechuic acid, hibiscus acid, quercetin, CGA, and catechin (Kebede et al., 2025). Research also supports the fact that fortification of fermented dairy products (e.g., yogurt, kefir) with anthocyanin-rich plant extracts, such as elderberry and riceberry rice, considerably increases the TPC of final product (Anuyahong et al., 2020; Bakir, 2025).

Table 12 Functional properties of Túró Rudi incorporated with roselle encapsulates

Treatment code	Fortification level (w/w)	TPC (mg GAE/g)	TMA (mg DSE/g)	DPPH (mg AAE/g)
R (control)	0%	3.87 ± 0.10^{b}	0.01 ± 0.00^{b}	0.87 ± 0.01^{b}
T1	2%	3.95 ± 0.03^{b}	0.02 ± 0.00^{b}	0.89 ± 0.00^{b}
T2	5%	4.08 ± 0.06^{b}	0.02 ± 0.00^{b}	1.18 ± 0.01^{a}
T3	9%	4.50 ± 0.03^{a}	0.05 ± 0.01^{a}	1.31 ± 0.01^{a}

Notes: Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different ($p>0.05$) at a 95% confidence level according to Tukey's HSD test

However, when comparing these results with TPC in the encapsulated powder, it highlights that producing Túró Rudi (T3) has resulted in a substantial boost in TPC (Figure 21), likely due to the impact of ingredients in both the curd matrix and coat with the dark chocolate.

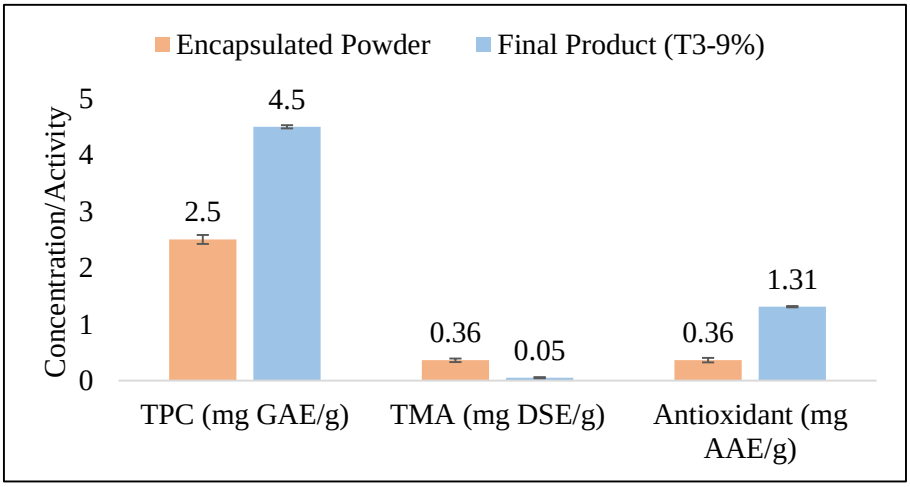


Figure 21 Bioactivity transition from encapsulated powder (with MD-to-GA ratio of 3:1) to Túró Rudi (9% enrichment). Error bars represent the SD of the mean.

For instance, control (3.87±0.10 mg GAE/g) already exceeds the encapsulated powder, confirming the contribution of dark chocolate in the TPC measurement. Dark chocolate is naturally rich in polyphenols, such as flavonols (Singh et al., 2024) and dark chocolate with 54-60% cocoa typically shows a TPC of 19.5 mg GAE/g (Ishak et al., 2024). Research also indicates that chocolates fortified with plant-based ingredients exhibit higher TPC than the additives alone because the cocoa powder/liquor formulated into the chocolates delivers a high phenolic baseline (Singh et al., 2024). Besides, there can be a considerable impact of wall material components, MD and GA, aiding in the release or stabilize of the phenolics from the chocolate matrix, in addition to the breaking of wall material and

roselle phenolics bonds during the extraction process before testing for TPC, making them more detectable.

Considering TMA, it is noticeable that TMA values remain low in Túró Rudi products, while a significant increase occurs at the 9% level (Table 12). Anthocyanins being highly unstable at pH conditions above 3.0, their presence in the final product confirms that the MD and GA-based wall material provided a strong structural barrier, preventing the roselle's red pigments from completely degrading during the mixing with the curd base, which typically has a pH of 5.2–5.8 that can potentially transform anthocyanins into colorless forms (Wu et al., 2018). On the other hand, the significant drop of TMA in the final product compared to the encapsulated powder (Figure 21), there is a chance that anthocyanins have bound to milk proteins in cheese through hydrophobic interactions (Jing and Giusti, 2005; Van de Langerijt et al., 2023), causing a masking effect, reducing the measurable anthocyanin concentration in standard tests (even during the extraction process before the test) even though they are physically existing. However, proteins were not extracted before the analysis of functional properties of Túró Rudi, marking it as a limitation of this study.

In contrast to TPC and TMA, the antioxidant activity indicates a significant increase starting at 5% fortification level (T2). This upsurge in antioxidant activity, even before the phenolic count increases (Table 12), suggestes that there can be both the contribution of dairy proteins (with inherent antioxidant activity) (Yang, 2024) and their synergistic effect

with roselle extract in enhancing the antioxidant profile of the final product. More particularly, dairy proteins in cheese can bind with phenolics in roselle encapsulated powder, potentially stabilizing them during processing (Wang et al., 2024b), causing a higher cumulative antioxidant value in T3 (1.31±0.01 mg AAE/g). Moreover, chocolate phenolics should also have contributed to the antioxidant capacity of the final product as the upsurge from encapsulated powder to Túró Rudi (T3) is too high (Figure 23), as well as because the control sample also has a high antioxidant activity (0.87±0.01 mg AAE/g), higher than the encapsulated powder.

All in all, despite the TMA drop, the fact that TPC and antioxidant activity remain high underscores that the 3:1 MD to GA wall efficaciously protected the core bioactive components during the Túró Rudi-making process. The film-forming protection provided by GA while MD ensuring the stable and dispersible powder properties (Cid-Ortega & Guerrero-Beltrán, 2020; Kurniati et al., 2025) have granted the superior protection for roselle bioactives within the chocolate coated cottage cheese matrix. Furthermore, functional properties reported in the final product demonstrates that it can deliver considerable doses of phenolics with antioxidant properties to the consumer.

4.6.2 Nutritional properties of Túró Rudi with roselle encapsulated powder

The nutritional analysis of Túró Rudi developed with added roselle encapsulated powder showed a steady rise in protein content, shifting from 14.66% (control) to 15.60% (T3) suggesting the encapsulated powder

contributes to additional amount of amino acids or nitrogenous compounds. This increase in protein content with higher fortifiactions of encapsulated powder can be due to the impact of GA, which is partially a glycoprotein, consisting of approximately 10% of arabinogalactan-proteins and hydroxyproline-rich glycoproteins (Qi et al., 1991). This finding aligns with the significant increase of the protein content in white jack bean tempe with increased levels of GA in a MD and GA matrix, as reported by Yarlina et al. (2023). On the contrary, adding more roselle encapsulates decreased fat content from 6.38% to 5.15%, which can be due to the replacement of a fraction of fat-rich cheese curd base with carbohydrate-based encapsulated powder representing an effective dilution of fat concentration in the final product. Moreover, with the increase of fortification level, moisture content decreased (however, was not significant, $p>0.05$), and total solid content increased ($p<0.05$), resulting in a denser or firmer texture in the cheese-based core of Túró Rudi. The effect of fortification on salt concentration was non-significant ($p>0.05$). Overall, the nutritional analysis demonstrated that incorporation of encapsulated roselle powder generated a cheese-based snack with higher protein and lower-fat contents compared to the typical Túró Rudi, without significantly affecting the moisture levels emphasizing the potential to utilize encapsulated powder in Túró Rudi for improved nutritional quality.

Table 13 Nutritional properties of Túró Rudi incorporated with roselle encapsulates

Treatment code	% of encapsulated powder (w/w)	Moisture (%)	Protein (%)	Fat (%)	Salt (%)	Total solids (%)
R (control)	0%	53.83±0.80^{a}	14.66±0.30^{b}	6.38±0.15^{a}	0.64±0.16^{a}	46.17±0.56^{b}
T1	2%	53.43±0.50^{a}	15.33±0.27^{ab}	5.99±0.42^{ab}	0.60±0.13^{a}	46.52±0.80^{b}
T2	5%	53.21±0.51^{a}	15.59±0.19^{a}	5.24±0.11^{b}	0.72±0.13^{a}	46.78±0.51^{ab}
T3	9%	52.25±0.59^{a}	15.60±0.26^{a}	5.15±0.20^{b}	0.93±0.18^{a}	47.75±0.59^{a}

Notes: Values denote the mean±SD of the three replicates based on one-way ANOVA. Means followed by the same superscript letter within the same column are not significantly different ($p>0.05$) at a 95% confidence level according to Tukey's HSD test

4.6.3 Sensory properties of Túró Rudi with roselle encapsulated powder

The color likeness tested with the 9-point hedonic scale analyzed with the Friedman test confirmed that there was no significant difference ($p>0.05$) between the treatments, including the reference. The median scores for the reference, T1, T2, and T3 were 7.0, 7.0, 6.5, and 7.5, respectively. Although there was no significance in the color likeness, when comparing the median score, it signifies that the low-level incorporation of roselle encapsulates generated a similar visual appeal to the Túró Rudi as the control, suggesting that T1 does not alter the original Túró Rudi color. Figure 22 illustrates how the three different treatments with varying levels of incorporated encapsulated roselle powder deviate from the reference, in terms of herbal flavor/tartness, red/pinkness and mouthfeel/softness (average scores were compared to obtain a more practical evaluation).

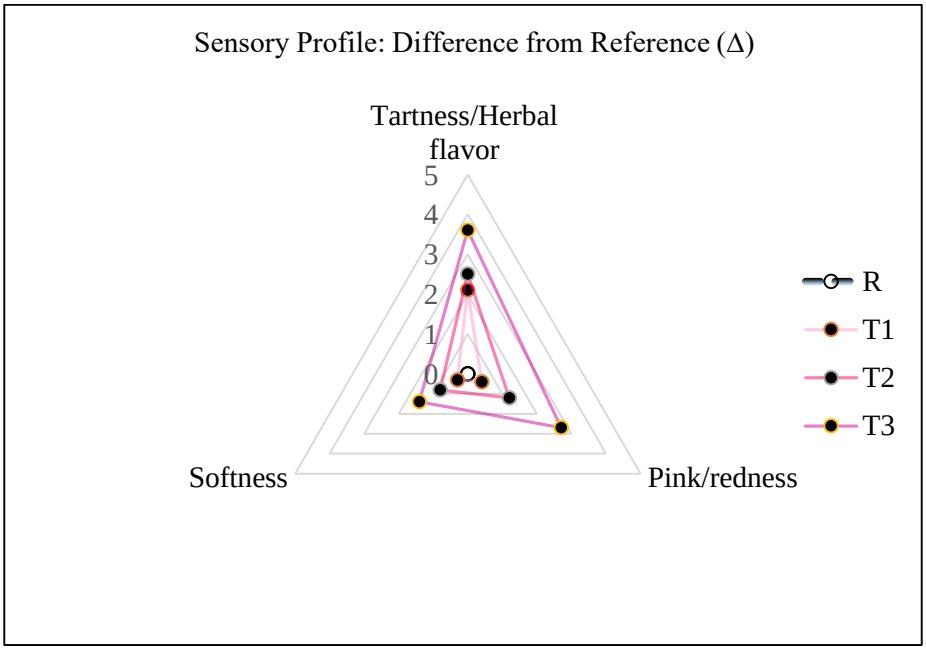


Figure 22 Specific sensory deviations of Túró Rudi with roselle encapsulates relative to the unfortified control baseline ("R" at the centre)

Accordingly, T1 recorded as average score of 2.1 for the tartness/herbal flavor, and 0.5 for both the color and softness. T2 showed a moderate balance in the medium-level incorporation of roselle-encapsulated powder into Túró Rudi, with average scores of 0.8 for softness, 1.2 for pink/redness, and 2.5 for tarness. T3 showed comparatively intense effects on tartness (3.6), softness (1.4), and pink/redness (2.7), suggesting a potent effect of this treatment on both chemical (flavor) and physical (pigment and texture) properties. However, based on the standard ANOVA run for the average score of each attribute, there was no significance ($p>0.05$) between the treatments for the tartness,

and softness. In contrast, significant pinkness/redness was observed in T3 compared with both T2 and T1 ($p<0.05$).

Among the tested specific attributes, “tartness” is scientifically linked to the concentration of organic acids, such as citric acid or malic acid, while “herbal” notes often stem from volatile components, such as phenolic compounds, aldehydes or terpenes, present in the extract. In roselle calyx, the principal compounds which cause tartness are hibiscus acid (13–24%), citric acid (12–20%), malic acid (2–9%), and tartaric acid (8%) (Da-Costa-Rocha et al., 2014). The significant increase in color intensity in T2 and T3 suggests a higher concentration of natural pigments, more particularly, anthocyanins, which are more stable in acidic pH levels. The simultaneous increase in tartness in T2 and T3, representing a more acidic environment, aligns with this improved redness. For instance, roselle infusions basically have a low pH (2.5-3.0), which can stabilize anthocyanins such as delphinidin-3-sambubioside, confirming the intense pink/redness (Da-Costa-Rocha et al., 2014; Avalos-Martínez et al., 2019). The relatively smaller shift in softness in roselle encapsulate-incorporated samples infers that while the chemical profile altered considerably, the physical matrix remained more stable.

In the final preference test analyzed with Chi-square test (based on equally preferred or not) T1, T2, and T3 received 1.63, 2.13 and 0.03 of contribution to Chi-square values, respectively. Although the Chi-square value (3.8) was not statistically significant ($p>0.05$) suggesting the observed specific distribution of ballots occurred by solely a random noise,

and the people actually liked all three treatments equally, T2 showed a strong descriptive lead in preference. Although T3 received the highest likeliness for its visual characteristics (in color likeliness question), the overall likeliness was very low, which can be due to the tartness. However, considering its enhanced functional and nutritional properties, improving its sensory acceptability by balancing with opposite tastes (e.g., strategic sweetening by adding honey or sugar/non-calorie sweeteners, salt adjustment, adding sodim bicarbonate as a buffer) (Purbowati et al., 2020) or by double encapsulating with lecithin or food-grade shellac/zein which delays the encapsulates get dissolved in saliva.

5. Conclusions

This research advances functional food development by systematically investigating plant polyphenol extraction for advanced ENC and their use in food products. The study focused on green solvents, specifically NADES, to extract phenolics from roselle calyx, followed by ENC and the creation of Túró Rudi, a fortified cheese-based dessert. Results showed that NADES, especially the Glu-LA-based system, offered an effective, food-grade, and sustainable alternative to conventional solvents for phenolic extraction. This method achieved higher or comparable yields of TPC (22.96±1.06 mg GAE/g), TMA (8.13±0.43 mg DSE/g), and antioxidant activity (13.90±0.80 mg AAE/g), while reducing safety and environmental risks linked to traditional solvents.

Using optimized extraction, ENC of the NADES roselle extract by freeze-drying with a 3:1 MD-to-GA matrix achieved the highest ENC

efficiency (99%), balanced powder yield (85%), and low moisture content (5.31%). The encapsulated powder stabilized and retained phenolics, especially under acidic conditions (pH 1-2), and exhibited free-flowing properties suitable for food use. Although some anthocyanins were lost during processing and storage, the freeze-dried ENC method preserved functional levels of bioactive compounds (2.50±0.08 mg GAE/g TPC and 0.36±0.04 mg AAE/g antioxidant activity), confirming freeze-drying as a viable option for heat-sensitive phenolics. The encapsulated extract was then incorporated into Túró Rudi, a cottage cheese-based Hungarian dessert, resulting in nutritional and functional improvements without compromising sensory quality. Higher fortification levels (up to 9%) increased protein content, reduced fat, and enhanced antioxidant activity (1.31±0.0 mg AAE/g), with elevated total phenolic content in the final product. The ENC matrix protected phenolics during processing, ensuring bioactive delivery in the dairy matrix. Sensory analysis showed that higher inclusion rates improved color and tartness without significantly affecting overall preference.

This study validated NADES as a green extraction technology for phenolic-rich plant materials and demonstrated that freeze-drying ENC effectively stabilizes sensitive phytochemicals, highlighting its potential in functional food development. Integrating green extraction, freeze-drying ENC, and product formulation produced a novel, phenolic-enriched dairy dessert with improved nutritional and functional properties, addressing gaps in current literature. Future research should enhance the stability and

bioavailability of encapsulated phenolics, scale up NADES-based extraction and ENC processes, and conduct *in vitro* and *in vivo* studies to assess health benefits in humans. This comprehensive approach supports the development of sustainable, health-promoting food ingredients and serves as a model for combining green chemistry with advanced food technology to valorize bioactive plant compounds.

6. Detailed summary

This section provides an overview of the dissertation by summarizing the key content and findings of each main chapter: introduction, literature review, methodology, results and discussion, and conclusion.

The introduction establishes the growing interest in polyphenols, especially CGAs and anthocyanins, as functional food ingredients due to their antioxidant, antimicrobial, and health-promoting properties. It highlights the challenges of polyphenol instability and poor bioavailability, particularly when incorporated into complex food matrices like dairy. The research objectives are set out clearly: to develop a green extraction protocol for polyphenols from roselle calyx and coffee beans along with HPLC-PDA analysis, optimize ENC parameters using food-grade excipients, apply the encapsulated extracts to fortify a popular dairy dessert (Túró Rudi), and to analyze its functional properties.

The literature review explores the role and significance of polyphenols—particularly CGAs and anthocyanins—in functional food applications. It discusses their health benefits, challenges related to stability and bioavailability, and their incorporation into dairy matrices. It

also briefly outlines the health effects of caffeine, an alkaloid present in coffee matrices. The review further examines extraction techniques for plant bioactives, focusing on the limitations of conventional organic solvents and highlighting the emerging use of NADESs as green, sustainable alternatives. The ENC of polyphenols, especially using freeze-drying methods and combinations of MD and GA as wall materials, is presented as a promising strategy to enhance stability, sensory properties, and functionality in food systems. The review also addresses the technological and sensory challenges of integrating polyphenol-rich extracts into dairy products, with a particular focus on the Hungarian dairy dessert, Túró Rudi as a model system.

Materials and methods chapter details the systematic experimental design implemented to achieve the study's objectives. It describes the plant materials (roselle calyx, green and roasted coffee beans), chemicals, equipments and food-grade ingredients used. Multiple extraction protocols were optimized, including methanolic, ethanolic, and NADES extraction methods, with variables such as pH, temperature, and solvent ratios carefully controlled. HPLC and spectrophotometric assays were employed to quantify CGA, caffeine, total phenolics, flavonoids, anthocyanins, and antioxidant activity of plant extracts. The ENC process using freeze-drying with varying MD-to-GA ratios is described in detail, followed by the formulation of Túró Rudi incorporating different levels of encapsulated roselle extract. The methods for evaluating functional, nutritional, and

sensory properties, as well as statistical analysis approaches, are also outlined.

The results revealed that optimized HPLC conditions enabled precise quantification of CGA in roselle extracts while both CGA and caffeine in coffee extracts. The results also demonstrate that NADES (particularly Glu-LA) provided superior extraction yields for phenolics, including anthocyanins, from roselle calyx compared to conventional solvents, while also being safer and more sustainable. The freeze-drying ENC process using a 3:1 MD-to-GA ratio achieved high ENC efficiency, favorable powder properties, and good color stability under acidic conditions. Incorporation of encapsulated roselle powder into Túró Rudi improved its nutritional profile—enhancing protein content, reducing fat, and significantly increasing antioxidant activity—while maintaining sensory acceptability, especially at moderate fortification levels. Sensory trials confirmed that higher inclusion levels increased pinkness, without significantly affecting the overall preference. The study highlights the synergy between dairy matrices and plant polyphenols, the effectiveness of freeze-dried ENC, and the practicality of using NADES for green extraction.

The dissertation concludes that integrating NADES-based extraction with freeze-drying ENC offers a sustainable, effective solution for stabilizing sensitive polyphenols in functional foods. The developed approach successfully produced a phenolic-enriched dairy dessert (Túró Rudi) with improved nutritional, functional, and sensory properties. The

ENC matrix protected bioactives during processing and storage, ensuring their delivery in the final product. The findings address gaps in the current literature on polyphenol stabilization and ENC and establish a foundation for further research on scaling up, optimizing bioavailability, and validating health benefits in human studies. This research therefore, serves as a model for leveraging green chemistry and advanced food technology to develop novel, health-promoting functional foods.

7. New scientific results

1. The optimal extraction parameters for roselle calyx were identified: The highest CGA yield from roselle calyx was achieved using an extraction pH of 7.0, 25% ethanol concentration, and 20 min extraction time when quantified by the HPLC-PDA method.
2. NADES solvent efficacy was defined for green coffee beans: Glu-Glyc-based NADES yielded competitive CGA (50.8±0.3 mg/g) and overall phenolic recovery (TPC: 48.7±0.6 mg GAE/g) to those of aqueous ethanol (CGA: 52.0±0.3 mg/g, TPC: 45.0±0.5 mg GAE/g), while offering superior sustainability and safety.
3. Superior green extraction was developed for phenolics from roselle calyx: Glu-LA-based NADES outperformed conventional ethanol in total phenolic yield (TPC: 22.69±1.06 mg GAE/g) and antioxidant activity (DPPH radical scavenging activity 13.9±0.80 mg AAE/g) at 50˚C, 30 min extraction, while resulting in comparable monomeric anthocyanins (TMA: 8.13±0.43 mg

DSE/g;), and CGA (2.50±0.02 mg/g) yields, making it an effective, superior green solvent.

4. Advanced ENC method was developed: The freeze-drying ENC method utilizing a 3:1 blend of MD to GA yielded a 99% ENC efficiency for polyphenol-rich green extracts of roselle calyx. The resulting excellent powder properties (free-flowing: 10%±0.00 Carr index and 1.11±0.00 Hausner ratio) low moisture (5.31%±0.00), quick water solubility (51±1.14 s), and excellent colour stability at pH 1-2, ideal for functional food systems.
5. Túró Rudi fortification was achieved: a dairy dessert (Túró Rudi) was developed by incorporating the encapsulated roselle extract powder at 2%, 5% and 9% fortification (calculated on a weight basis relative to the curd-sugar base mass). Higher level fortification (9%) resulted in 4.50±0.03 mg GAE/g of TPC, 0.05±0.04 mg DSE/g of TMA and 1.31±0.01 mg AAE/g of antioxidant activity.

8. Publications

8.1 Journal articles (Peer-reviewed indexed journals)

UR Chandimala, Zsolt Ajtony, Beatrix Sik, (2024). Citrus flavonoids (naringin and hesperidin) as functional ingredients in dairy products, Bio Web of Conferences, 125, 02004. https://doi.org/10.1051/bioconf/202412502004

UR Chandimala, Zsolt Ajtony, (2025). Application of chlorogenic acid in dairy product enrichment/fortification – a review, LWT-Food Science & technology, 232. https://doi.org/10.1016/j.lwt.2025.118416 (Q1, D1)

UR Chandimala, Ottó Dóka, (2026). Rheological analysis of dairy products: Instrumentation and Applications to Functional Dairy Products Enriched with Plant-based Ingredients, International Journal of Food Studies (Accepted to publish) (Q3)

UR Chandimala, Zsolt Ajtony, (2026). Green Extraction and Advanced Encapsulation of Roselle (*Hibiscus sabdariffa* L.) Phenolics: Harnessing Natural Deep Eutectic Solvents for Sustainable Nutraceutical Applications, Green Analytical Chemistry (Under review) (Q1)

8.2 Journal articles (Peer-reviewed non-indexed journals)

UR Chandimala, Beatrix Sik, Zsolt Ajtony, (2025). An Experimental Design for the Analysis of 5-Caffeoylquinic Acid (5-CQA) in Ethanolic Extracts of Hibiscus (*Hibiscus sabdariffa* L.) Flower, Georgikon for Agriculture, 29, 1-16. https://doi.org/10.70809/6561

UR Chandimala, Zsolt Ajtony, (2025). Quantification of Caffeine and 5-Caffeoylquinic Acid (5-CQA) in Seven European Commercial Coffee (Coffea arabica) Types Using HPLC-PDA, Journal of Agro-Technology and Rural Sciences, 5(1), 1-5. https://doi.org/10.4038/atrsj.v5i1.71

UR Chandimala, Beatrix Sik, Zsolt Ajtony, (2026). The Potential of Natural Deep Eutectic Solvents for the Extraction of Phenolic Compounds and Caffeine from Green Coffee (Coffea arabica) Beans, Acta Agronomica Óváriensis, 67(1). https://doi.org/10.17108/ActAgrOvar.2026.67.1.90

UR Chandimala, Beatrix Sik, Zsolt Ajtony, (2026). Developing a dairy dessert (Túró Rudi) with freeze-dried encapsulated powder of roselle (*Hibiscus sabdariffa* L.) calyx, Advances in Food Sciences (accepted to publish)

8.3 Conferences attended

The 6th National Symposium on Agriculture, Eastern University, Sri Lanka, 2024.03.12-oral presentation on "A mini-review: incorporating encapsulated polyphenols in meat products".

The 10th International Conference on Agricultural and Biological Sciences, Szechenyi Istvan University, 2024.07.25-oral presentation on "Citrus flavonoids (naringin and hesperidin) as functional ingredients in dairy products".

XXXIV Keszthely Plant Protection Forum, MATE Georgikon Capus, 2025.01.16-oral presentation on "An Experimental Design for the Analysis of 5-Caffeoylquinic Acid (5-CQA) in Ethanolic Extracts of Hibiscus (*Hibiscus sabdariffa* L.) Flower"

International Symposium on Agro-Technology and Rural Sciences, University of Colombo, Sri Lanka, 2025.06.26-oral presentation on "Quantification of Caffeine and 5-Caffeoylquinic Acid (5-CQA) in Seven European Commercial Coffee (*Coffea arabica*) Types Using HPLC-PDA"

40th óvár scientific day international conference, 2025.11.12-oral presentation on "Assessing the Potential of Natural Deep Eutectic Solvents (Glucose and Glycerin) for the Extraction of Phenolic Compounds and Caffeine from Green Coffee Beans"

Agriculture and Food conference, Bulgaria 2026.08.10-13-oral presentation on “A review: application of plant phenolics in meat products”

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11. APPENDICES

11.1 Sensory ballot sheet

Sensory Evaluation Ballot: Túró Rudi with Roselle Extract

Panelist ID: _____ Date: _____ Gender: _____

Part 1: Visual Assessment

First, look at the samples with the 4-digit codes. Rate how much you like the **color** of each sample.

1 = Dislike Extremely | 2 = Dislike Very Much | 3 = Dislike Moderately | 4 = Dislike Slightly | 5 = Neither Like nor Dislike 6 = Like Slightly | 7 = Like Moderately | 8 = Like Very Much | 9 = Like Extremely

1. How much do you like the color of each sample?

Sample Code	1	2	3	4	5	6	7	8	9
Reference	☐	☐	☐	☐	☐	☐	☐	☐	☐
0141	☐	☐	☐	☐	☐	☐	☐	☐	☐
0410	☐	☐	☐	☐	☐	☐	☐	☐	☐
0014	☐	☐	☐	☐	☐	☐	☐	☐	☐

Part 2: Comparison with control

Taste the identified **Control (Reference) first.** Then, evaluate the 4-digit samples (questions 2-4). Use the water provided between each taste.

2. In each treatment, indicate how intense the **herbal flavor/tart or sour taste** was **compared to the Control** (using the scale given below)

0 = No Difference | 1 = Very Slight | 2 = Slight | 3 = Moderate | 4 = Intense/large | 5 = Very intense/large

Sample Code	0 (no difference)	1	2	3	4	5 (very intense)
0141	☐	☐	☐	☐	☐	☐
0410	☐	☐	☐	☐	☐	☐
0014	☐	☐	☐	☐	☐	☐

3. In each treatment, indicate how intense the **pink/redness** was compared to the Control (using the scale given below)

0 = No Difference | 1 = Very Slight | 2 = Slight | 3 = Moderate | 4 = Intense/large | 5 = Very intense/large

Sample Code	0 (no difference)	1	2	3	4	5 (very intense)
0141	☐	☐	☐	☐	☐	☐
0410	☐	☐	☐	☐	☐	☐
0014	☐	☐	☐	☐	☐	☐

4. In each treatment, indicate how you perceived the **texture of the core (softness)** compared to the Control using the scale given below;

0 = No Difference | 1 = Slightly softer | 2 = Moderately softer | 3 = Softer | 4 = Much softer | 5 = Too soft/mushy

Sample Code	0 (No difference)	1	2	3	4	5 (Too soft/mushy)
0141	☐	☐	☐	☐	☐	☐
0410	☐	☐	☐	☐	☐	☐
0014	☐	☐	☐	☐	☐	☐

Part 3: Final Preference

5. Which of the four treatments did you prefer most?

Sample Code: _____

Why? (e.g., best balance, closest to control, unique herbal note)

_____

_____

_____