Optimization of extraction using response surface methodology and chromatographic characterization of phenolics and flavonoids from plants

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ABSTRACT

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Empetrum nigrum is a dwarf evergreen shrub rich in phenolics. Among these compounds, batatasin-III is a topic on which comprehensive research is increasing due to its significant impact on the ecosystem. It can affect vegetation growth through a characteristic allelopathic properties. Although reindeer are known to live in the habitat of *Empetrum nigrum*, they generally avoid consuming this plant. However, DNA from *Empetrum nigrum* found in faecal samples has raised questions about the reindeer's diet and grazing practices. Furthermore, little is known about how phenolic and flavonoid compounds are presented and metabolized after digestion. Therefore, optimizing extraction conditions for accelerated solvent extraction (ASE) to recover phenolic and flavonoid compounds from *Empetrum nigrum* and reindeer faeces is studied.

This thesis aims to systematically optimize the extraction conditions for ASE. Additionally, gas chromatography-mass spectrometry (GC-MS) analysis was applied to discover the metabolic profile of different parts of *Empetrum nigrum*, including young and old shoots and litter, and reindeer faeces and to assess the presence and content of batatasin-III. The variability and interaction between the factors under study are expressed through response surface methodology (RSM). Extraction results were evaluated based on total phenolic content (TPC) and total flavonoid content (TFC). The matrix patterns were verified by extraction under optimized conditions. TPC and TFC were determined by UV-Vis spectroscopy, while results related to metabolite composition and batatasin-III content were obtained using GC-MS.

The optimization models gave good predictive results and were statistically verified. Young shoots contained the highest concentrations of TPC and TFC, followed by older shoots, litter, and reindeer faeces. GC-MS analysis also captured various compound groups present in *Empetrum nigrum*. Batatasin-III was also detected at approximately 10.7 mg/g DW in young and older shoots. These concentrations were significantly lower in litter and reindeer faeces, at 0.76 mg/g DW and 0.4 mg/g DW, respectively.

These findings demonstrate the potential of applying ASE methods to the recovery of organic compounds. Furthermore, batatasin-III persists after digestion by herbivores, which is evidence that reindeer may have accidentally consumed *Empetrum* leaves. However, more research is required. To our knowledge, this is the first study on batatasin-III in reindeer faeces, offering new insights into the metabolism of post-digestion bioactive compounds and the herbivorous-plant relationship in the northern region. .

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SYMBOLS AND ABBREVIATIONS

Roman characters

A_o	constant term	
A_i	linear parameters coefficients	
A_{ii}	quadratic parameters coefficients	
A_{ij}	interaction parameters coefficients	
$C_{phenolic\text{-}plant}$	Concentration of phenolic compounds obtained from calibration curve of plant matrix	mg/L
$C_{phenolic\text{-}reindeer}$	Concentration of phenolic compounds obtained from calibration curve of reindeer faeces matrix	mg/L
$C_{flavonoid\text{-}plant}$	Concentration of flavonoid compounds obtained from calibration curve of plant matrix	mg/L
$C_{flavonoid\text{-}reindeer}$	Concentration of flavonoid compounds obtained from calibration curve of reindeer faeces matrix	mg/L
k	number of variables	
TPC_{plant}	Total phenolic content of plant matrix	mg GAE / g DW
$TPC_{reindeer}$	Total phenolic content of reindeer faeces matrix	mg GAE / g DW
TFC_{plant}	Total flavonoid content of plant matrix	mg RE / g DW
$TFC_{reindeer}$	Total flavonoid content of reindeer faeces matrix	mg RE / g DW
V	volume of extract	L
m	mass of the dried samples	g
x_i	variables	

Greek characters

ε	residual associated to the experiments

Abbreviations

ANOVA	Analysis of variance
ASE	Accelerated solvent extraction
BBD	Box-Behnken design
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
DF	Degree of freedom
DoE	Design of experiments
DW	Dry weight
GAE	Gallic acid equivalence
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectroscopy
MAE	Microwave-assisted extraction
MS	Mass spectroscopy
RE	Rutin equivalence
RSM	Response surface methodology
RT	Retention time
SIM	Selected ion monitoring
SS	Sum of squares
TFC	Total flavonoid content
TMCS	Trimethylchlorosilane

TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
UV-Vis	Ultraviolet-Visible

Table of contents

Abstract

Acknowledgements

Symbols and abbreviations

Declarations

1 Introduction 13
2 Background knowledge 16
2.1 Empetrum nigrum 16
2.1.1 General information 16
2.1.2 Phytochemical composition 17
2.2 Reindeer faeces 18
2.3 Extraction technologies for organic compounds 19
2.3.1 Ultrasonic-assisted extraction (UAE) 19
2.3.2 Microwave-assisted extraction (MAE) 19
2.3.3 Accelerated solvent extraction (ASE) 20
2.4 Design of experiments (DoE) 21
3 Materials and methods 23
3.1 Materials 23
3.2 Methods 24
3.2.1 Sample preparation 25
3.2.2 Accelerated solvent extraction (ASE) 26
3.2.3 Experimental design 26
3.2.4 Total phenolic content (TPC) determination 28
3.2.5 Total flavonoid content (TFC) determination 29
3.2.6 Gas chromatography – Mass spectroscopy (GC-MS) 30
4 Results and discussion 32
4.1 Sample pretreatment 32
4.2 Single-factor experiments 33

4.2.1 Plant matrix 33
4.2.2 Reindeer faeces matrix 37
4.3 Box-Behnken design 41
4.3.1 Plant matrix 41
4.3.2 Reindeer faeces matrix 50
4.4 Sample extractions 57
4.4.1 Model validation 57
4.4.2 Sample extraction results 58
4.5 GC-MS 60
4.5.1 *Empetrum nigrum* profiling 60
4.5.2 Reindeer faeces profiling 64
4.5.3 Batatasin-III concentrations 66
4.6 Limitations and recommendations 70
5 Conclusion 72
References 74

Appendix

List of figures

Figure 1: Diagram of workflow

Figure 2: Investigated condition effects on TPC of plant matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

Figure 3: Investigated condition effects on TFC of plant matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

Figure 4: Investigated condition effects on TPC of reindeer faeces matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

Figure 5: Investigated condition effects on TFC of reindeer faeces matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

Figure 6: *Empetrum* extracts throughout 15 experimental extractions

Figure 7: 3D contour of TPC of *Empetrum* matrix

Figure 8: 3D contour of TFC of *Empetrum* matrix

Figure 9: 3D contour of TPC of reindeer matrix

Figure 10: 3D contour of TFC of reindeer matrix

Figure 11: Metabolites profiling of *Empetrum nigrum*

Figure 12: Calibration curve of batatasin-III

Figure 13: Chromatograph plot between young shoots (black), old shoots (red), and litter (green)

Figure 14: Calibration curve of batatasin-III under SIM mode

List of tables

Table 1: Examples of research for optimizing ASE procedure applied in plant matrices

Table 2: Summary of investigated samples

Table 3: List of chemicals used and their specifications

Table 4: Single factor experiments design

Table 5: Box-Behnken experimental design

Table 6: Sample pretreatment results

Table 7: TPC and TFC of *Empetrum* matrix under investigated conditions

Table 8: TPC and TFC of reindeer faeces matrix under investigated conditions

Table 9: Box-Behnken design for *Empetrum nigrum* matrix

Table 10: ANOVA summary table according to TPC and TFC responses of *Empetrum* matrix coefficients of model equation, sum of squares (SS), degrees of freedom (DF), mean squares (MS), F statistic (F-value), and probability value (p-value)

Table 11: Optimal extraction conditions of *Empetrum nigrum* regarding to TPC and TFC

Table 12: Box-Behnken design for reindeer faeces matrix

Table 13: ANOVA summary table according to TPC and TFC responses of reindeer matrix with coefficients of model equation, sum of squares (SS), degrees of freedom (DF), mean squares (MS), F statistic (F-value), and probability value (p-value)

Table 14: Method validation results

Table 15: TPC and TFC of different matrices

Table 16: Metabolites detected in *Empetrum nigru*m young shoots

Table 17: Additional metabolites detected in old shoots

Table 18: Metabolites detected in reindeer faeces matrix

Table 19: Batatasin-III concentration in different matrices

DECLARATIONS

Turnitin

The originality of this thesis has been reviewed with the Turnitin similarity checking service.

AI usage

The author of the thesis, Chau Nguyen, used the following AI-tools during the preparation of the thesis:

1. Chatgpt
    a. Purpose of use: Support in suggesting information sources, language refinement.
    b. Explanation of the use of the tool: The AI was used to search for relevant information sources and scientific articles. The information was then checked against the Scopus database and other authoritative databases. The AI may used to refine and suggest alternative sentence structure for describing the data.

Responsibility

The author, Chau Nguyen, takes full responsibility for the content of this thesis and has reviewed and edited the content generated by the possible use of AI tools.

1 Introduction

Bioactive compounds, specifically phenolics and flavonoids, are playing a vital role in the pharmaceutical, cosmetic, and functional food industries. They possess superior antioxidant, antimicrobial, anti-inflammatory, and therapeutic properties, attracting considerable attention. Recently, there has been a growing trend towards using plant-derived products, evidenced by the rapid growth of the global polyphenol market, valued at approximately US$1.7 billion in 2022 and projected to reach nearly US$3 billion by 2030 (Grand View Research, n.d.). Among plant species, *Empetrum nigrum* is a promising candidate for extraction, with numerous studies on its active compounds and evidence of its abundant phenolic content. In addition to its rich phytochemical content, the species is of high ecological importance. It is abundant in boreal and Arctic ecosystems, creating dense vegetation and dominating over other vegetation in northern part of its range (González et al 2014). Its ability to form near monocultures is largely attributed to its longevity, dense clonal growth and the production and accumulation of the bioactive phenolic compound, batatasin-III.

Reindeer and caribou (*Rangifer tarandus*) are key herbivores across Arctic tundra ecosystems. Reindeer grazing alters the abundance of dwarf shrubs and the composition of the plant communities (Väisänen et al 2013). Reindeer are thought to avoid *Empetrum*-dominated areas (Iversen et al 2014) and select against *Empetrum* in their diets owing to the plant's high phenolic and low nutrient content (Zamin et al. 2017, González et al 2014). Yet, they inevitably graze also areas dominated by phenolic-rich plants and ingest them, whereby bioactive compounds from these plants can enter the digestive system, impact their nutrient uptake (Zamin et al. 2017), and also be quantifiable in faeces. While reindeer diets have been found to contain *Empetrum* DNA, it is unclear if this is because of ingestion of leaves or of berries, which are nutrient-rich and do not contain batatasin-III (Lavola et al. 2017). Therefore, studying reindeer faecal matrix is a promising topic for ecological vegetation, offering an opportunity to examine the detectability and stability of bioactive compounds after passing through a ruminant digestive system. In particular, the detection of batatasin-III in the matrix could assess the transfer and transformation of certain phenolic compounds. Furthermore, as mentioned above regarding the growth-inhibiting properties of batatasin-III, since reindeer faeces is directly excreted into the soil, the remaining phenolic compounds

may partially influence nutrient and chemical cycles in the soil environment, leading to the impact on the growth of some plant species. However, there are currently few studies regarding the appearance and recoverability of phenolic compounds from reindeer faeces.

The choice of extraction method is crucial for a comprehensive analysis of the chemical composition within the matrix. In recent years, along with technological advancements and the trend towards sustainable development, there has been increasing attention towards sustainable extraction technologies, with ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) being the most frequently mentioned. UAE offers the benefits in reducing extraction time and lowering solvent usage while maintaining the efficiency of recovering target compounds (Gao et al 2021). Meanwhile, MAE uses radiation to increase temperature and pressure within the sample, which disrupts cell structure and releases bioactive compounds. Similarly, MAE has been shown to be an efficient extraction method with low extraction time and solvent usage (Roberts et al 2019). However, both techniques have certain limitations. The UAE and MAE methods often require specialized equipment with relatively high initial investment costs. Furthermore, the transfer of optimization processes from laboratory to industrial scale remains challenging. Moreover, further comparative studies between extraction methods are needed to comprehensively evaluate their effectiveness.

Therefore, in this thesis, accelerated solvent extraction (ASE) has been studied and optimized. ASE is considered as an advanced extraction technique for capturing bioactive compounds from solid samples because it allows control of extraction conditions, lowers solvent consumption, and improves extraction efficiency compared to conventional methods. When combined with gas chromatography-mass spectrometry (GC-MS), ASE provides a promising analytical method for identifying and characterizing phenolic and flavonoid compounds in complex chemical substrates. Furthermore, although ASE has been frequently employed in the extraction of various plant species in the last 10 years, the optimization of conditions for recovering phenolics and flavonoids from *Empetrum* and reindeer faeces has yet to be studied.

In summary, there remains a knowledge gap regarding the optimization of ASE operating parameters for the recovery of phenolic and flavonoid compounds from the *Empetrum nigrum* plant and reindeer faece matrices. Moreover, detailed qualitative and quantitative characterization of batatasin-III was provided by GC-MS analysis. Filling this research gap

contributes to the comprehensive development of a more sustainable and effective extraction method for complex sample matrices. Besides, studying phenolic compounds in plant and animal faeces samples provides a better understanding of their metabolism and persistence after digestion. To achieve the stated aim, the objectives of this research are:

1. To investigate how solvent concentration, extraction temperature and time affect the ASE extraction method and optimize the process for above-ground parts of *Empetrum nigrum* and reindeer faeces to recover phenolic and flavonoid compounds.
2. To calculate the total phenolic content (TPC) and total flavonoid content (TFC) parameters of both matrices, which is an evaluation for the extraction efficiency of the investigation.
3. To qualitative determination of phenolic and flavonoid compounds in the extract solution by ASE-optimized method using GC-MS.
4. To evaluate the presence and quantification of batatasin-III in the extracts of the two matrices and compare the results between them.

2 Background knowledge

This chapter demonstrated the foundational knowledge of the main topic of the study. It introduces the importance of the black crowberry (*Empetrum nigrum*) and reindeer faeces in ecology, and common extraction technologies for recovering phenolic compounds from complex matrices.

2.1 Empetrum nigrum

Empetrum nigrum is a widely distributed evergreen dwarf shrub in northern regions (Ekaterina et al 2021). This species is attracting considerable academic attention due to its abundance of phenolic compounds and its characteristic growth-inhibiting properties (González et al 2014, Ekaterina et al 2021). *Empetrum nigrum* is rich in bioactive compounds with antioxidant, antimicrobial, and phytotoxic properties (Ekaterina et al 2021). Batatasin-III, one type of phenolic compounds found in *Empetrum*, and its allelopathic (Hierro et al 2021) properties is also a subject of interest, with numerous studies focusing on the aspect. Studies by Nilsson & Wardle (2005) and González et al (2014) highlight *Empetrum*'s importance in ecosystems, as it contributes in the nutrient cycle in soil and the growth of other plant species, as well as for pasture quality for semi-domesticated reindeer (Tuomi et al 2024).

2.1.1 General information

Empetrum nigrum belongs to the family Empetraceae; however, based on chemotaxonomic and molecular genetic similarity, there is still considerable debate regarding the precise classification of this species. *Empetrum nigrum*, known as black crowberry, is a plant commonly found throughout Russia, Japan, China, and Europe. It often forms dense, dominant vegetation covering large areas of Arctic tundra. It can tolerate very harsh environmental (abiotic) conditions, but it can also take advantage of more favourable conditions through increased growth rates. (Ekaterina et al 2021)

Furthermore, *Empetrum nigrum* interacts strongly with herbivorous animals such as reindeer. Grazing pressure can alter the population and chemical composition of the phenolic compounds in crowberries, thereby affecting ecosystem dynamics and the interactions between plants and herbivores (Väisänen et al 2013). Therefore, *Empetrum nigrum* is an important ecological model species for studying the relationship between secondary metabolites and ecosystem function.

2.1.2 Phytochemical composition

Empetrum nigrum has an extremely rich phytochemical composition, mainly consisting of phenolic compounds, flavonoids, tannins, anthocyanins, and stilbene derivatives (Ekaterina et al. 2021). These compounds have been widely researched and employed in many fields due to their antibacterial, antioxidant, and allelopathic properties (Ekaterina et al. 2021, Lavola et al 2017). In particular, phenolic compounds are observed to be concentrated in extracts from the fruit and above-ground parts of *Empetrum* (Kähkönen et al. 2001).

Among the identified compounds, batatasin-III, or 3,3′-dihydroxy-5-methoxybibenzyl, has attracted considerable attention due to its characteristic phytotoxicity and allelopathic properties. Previous studies have demonstrated that batatasin-III restricts seed germination and root elongation in many vascular plant species (Tybirk et al 2000), suggesting that this compound may contribute to the competitive success and ecological dominance of *Empetrum nigrum* in tundra ecosystems. However, although batatasin-III is phytotoxic to all tested species in bioassays, its effects in natural systems are strongly influenced by the environmental context (Ryde et al 2025) and cannot alone account for allelopathic activity. Therefore, batatasin-III concentration alone cannot fully explain the growth-inhibiting effects associated with *Empetrum nigrum*, highlighting the complexity of plant chemistry interactions in nature. (González et al 2014)

The phytochemical composition of *Empetrum nigrum* and other tundra plant species may change due to environmental conditions, nutrient availability, and increased grazing intensity (Zamin et al 2017, Väisänen et al 2012). Väisänen et al. (2012) noted that global warming and reindeer grazing altered the concentrations of phenolic compounds in *Empetrum* as well as their growth in the environment. These findings emphasized the significance of accurate methods of quantifying batatasin-III and phenolics in general: they

will allow for studying how bioactive phenolic compounds in dominant plants vary with their environment and with a warming climate (Zamin et al 2017).

2.2 Reindeer faeces

The reindeer *Rangifer tarandus* is one of the herbivorous animals in the ecosystems of the Arctic and sub-Arctic regions. Through grazing, trampling, and nutrient redistribution, reindeer influence vegetation composition (Olofsson et al., 2004). Because *Empetrum* makes up to 60% of plant biomass in tundra pastures, reindeer are likely to ingest them even if they would not select for them in their diets (Zamin et al 2017). These secondary metabolites of ingested plants can enter the digestive system and then be excreted in faeces. Therefore, reindeer faeces can be considered a biotransformed environmental matrix containing partially decomposed materials and residual plant metabolites. The composition of the faecal pellets may reflect the food, the gut microbiota, and its metabolism, and is a potential matrix for studying the transfer of nutrients after digestion.

Compared to plant samples, faecal samples are predicted to present significant challenges for both extraction and bioactive compounds detection due to their chemical complexity, heterogeneity, and high organic matter content. Components such as proteins, lipids, and fibre can interfere with the extraction and detection of phenolic compounds. Furthermore, the consumption time of phenolic-rich plants of the reindeer and the remaining bioactive compounds after digestion in the faeces samples used in this study is difficult to control. Therefore, an optimal and reliable extraction procedure is needed. Phenolic concentrations in the faeces samples are also predicted to be much lower than in the *Empetrum* plant matrix.

In summary, studying residual phenolic compounds in reindeer faeces is ecologically important because it provides information about the persistence and redistribution of organic compounds. In particular, the discovery of compounds like batatasin-III helps us understand the trophic transport, persistence and longevity, and their potential re-emergence in the soil through animal excretion.

2.3 Extraction technologies for organic compounds

To accurately analyse the chemical properties of plants, having suitable extraction methods for capturing the phenolic and flavonoid compounds from biological matrices is one of the most important factors. There are many elements such as solvent polarity, temperature, operating time, and sample composition that significantly impact the extraction efficiency (Dai and Mumper, 2010). For decades, maceration and Soxhlet extraction have been dominant and applied from small-scale to industrial levels. However, there are several drawbacks regarding to the extraction time, solvent, and energy consumption.

Aiming to achieve sustainable development while maintaining high efficiency, many modern extraction technologies have been researched. Among these alternative methods, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and solvent-accelerated extraction (ASE) are commonly applied, especially in the analysis of phenolic compounds.

2.3.1 Ultrasonic-assisted extraction (UAE)

Plant cell walls are broken down by ultrasound waves from UAE, and the solvent permeability into the sample is improved (Chemat et al 2017). This method allows for working efficiently with shorter extraction times and smaller amounts of solvents compared to traditional techniques (Gao et al 2021). Recent studies have successfully proved the effective application of UAE for extracting phenolics and flavonoids from organic and plant matrices because it is simple to operate and has a lower operational cost (Chemat et al 2017).

2.3.2 Microwave-assisted extraction (MAE)

Solvent and sample matrix were rapidly heated by microwave energy in the MAE method, thereby enhancing the release of intracellular compounds (Tatke and Jaiswal 2011). Rapid extraction and reduced solvent usage are among the advantages offered by MAE. However, the high temperatures generated during microwave processing can degrade some heat-sensitive organic compounds.

2.3.3 Accelerated solvent extraction (ASE)

Among advanced extraction technologies, accelerated solvent extraction (ASE) has been considered as an efficient method for recovering organic compounds from complex matrices. ASE is an advanced method developed from the traditional Soxhlet extraction method. However, compared to this exhaustive extraction method, ASE offers several superior advantages. ASE operates under high temperature and high pressure conditions, allowing the solvent to remain in a liquid state throughout the extraction process, even at temperatures above its boiling point (Richter et al 1996). Increasing temperature enhances the solubility and diffusion rate of the analyte, while the pressure facilitates the osmosis of the solvent into the sample. As a result, ASE has high extraction efficiency with short extraction times and minimal solvent usage.

ASE has been widely studied and applied in numerous scientific papers with different plant matrices, listed in the table 1. This method is particularly suitable for complex matrices such as plants because it can control precisely the extraction operational setup, such as solvent concentration, temperature, and extraction time.

Table 1: Examples of research for optimizing ASE procedure applied in plant matrices

No	Research	Investigated conditions	References
1	"Optimization of ASE procedure for grape skin antioxidants using Box-Behnken design"	Ethanol concentration (%), temperature (°C), Time (minutes)	Li et al 2019
2	"Maximizing recovery of phenolic content wild strawberry leaves using MAE and ASE"	Solvent-to-sample ratio, temperature (°C), Time (minutes)	Terpinc et al 2023
3	"Optimization of ASE for Yaobitong capsule for eight active compounds"	Methanol concentration (%), temperature (°C), Time (minutes), flushing volume (%)	Ji et al 2020
4	"Accelerated solvent extraction of bioactive compounds from carrot"	Number of cycles (cycles), temperature (°C), Time (minutes)	Vanja et al 2018
5	"Optimization of Accelerated Solvent Extraction of Phenolic Compounds from Chaga Using Response Surface Methodology"	Ethanol concentration (%), temperature (°C)	Weaam et al 2022

The extraction efficiency of ASEs is significantly dependent on operating conditions. Solvent polarity determines the solubility of target compounds, while extraction temperature affects analyte diffusion and matrix disruption. Similarly, extraction time affects the degree of compound recovery, although excessive extraction conditions can promote degradation of sensitive metabolites. Therefore, optimizing ASE parameters is necessary to maximize the recovery of phenolics and flavonoids while minimizing degradation and solvent usage.

In this study, ASE was chosen as the primary extraction technique due to its reported efficiency, reproducibility, and suitability for complex media matrices. This method was applied to both the above-ground tissue of *Empetrum nigrum* and reindeer faeces to evaluate the recovery of phenolic compounds, including batatasin-III, from different biological samples.

2.4 Design of experiments (DoE)

Design of experiment (DoE) is a widely used mathematical statistical method for evaluating the influence of multiple experimental variables on one or more response variables. DoE simplifies the research process by minimizing the number of experimental runs compared to traditional methods. Therefore, DoE is widely applied in optimizing extraction processes.

Optimizing extraction processes often involves operational parameters such as solvent concentration, extraction time, working temperature, solvent/sample ratio, and so on. However, these parameters can often have significant interactions among them and together affect the extraction efficiency in a non-linear way (Wang et al. 2022). Therefore, relying solely on traditional methods of testing each parameter individually may not yield a truly optimal point.

Among the design methods, response surface methodology (RSM) is a regularly applied technique when optimizing extraction processes. Optimizing a system affected by many individual variables is considered highly effective when applying this method. Currently, quite a few studies have utilized this approach in optimizing extraction conditions for phenolic and flavonoid compounds.

RSM typically generates polynomial models to predict the relationship between extraction parameters. These models are then often represented through contour plots and 3D response

surface plots, providing a clearer picture of the interactions and optimal extraction conditions. In addition to minimizing experimental effort, the reproducibility of the method and the statistical reliability of the experiments are also demonstrated by this approach.

The Box-Behnken design (BBD) is considered one of the most broadly employed RSM designs for process optimization (Bezerra et al 2008). This model evaluates variables at three levels: low (-1), central (0), and high (1). It arranges special experimental models to evaluate the interaction between variables without running experiments at extreme values. In extraction process optimization studies, BBD has been successfully applied to find the optimal points for extraction time and temperature, volume of solvent, and solvent concentration (Zhang et al. 2021).

In this research, the BBD method was utilized to optimize the ASE parameters to achieve the highest phenolics and flavonoids concentration from *Empetrum* and reindeer faeces. Solvent concentration, working temperature, and time are the three investigated factors. The response variables for evaluating extraction efficiency were total phenolic content (TPC) and total flavonoid content (TFC), respectively. The experimental results were fitted into equation (1). (Bezerra et al 2008)

$$y = A_0 + \sum_{i=1}^{k} A_i x_i + \sum_{i=1}^{k} A_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} A_{ij} x_i x_j + \varepsilon \quad (1)$$

With k is the number of variables, A_0 is the constant term, A_i represents the linear parameter coefficients, A_{ii} represents the quadratic parameter coefficients, A_{ij} represents the interaction parameters coefficients, x_i acts for the variables, and ε is the residual associated to the experiments.

The application of RSM has enabled the systematic optimization of ASE conditions, while also allowing the study of potential interactions between extraction parameters.

3 Materials and methods

The chapter views the materials, instruments, extraction optimization procedures, and experimental design applied in the thesis. A methodology was designed to evaluate the extraction efficiency of phenolic and flavonoid compounds from above-ground parts matrices of *Empetrum nigrum* and reindeer faeces using accelerated solvent extraction (ASE) based on total phenolic content (TPC) and total flavonoid content (TFC). Moreover, GC-MS analysis to determine the profile of phenolics and flavonoids of these matrices will also be presented below. Among those compounds, batatasin-III is the target substance and its concentration within the matrices will be quantified using the same analytical method.

3.1 Materials

The plant material used in this study included above-ground parts of *Empetrum nigrum* at two stage, including old and young shoots, and its litter. *Empetrum nigrum* plant matrices were harvested at Stallogargo between July 4th and July 7th. Plant samples were frozen at -20°C until used for extraction. All the samples were freeze-dried before extraction to reduce moisture content and improve sample stability. Reindeer faeces were harvested at Stallogargo shore on July 7th and processed similarly to the plant samples.

Before extraction, all samples were finely ground into powder using a mechanical grinder to ensure matrix homogeneity. The homogenized samples were stored in airtight containers in a freezer until the next analysis.

Table 2 summarizes the plant and reindeer faeces samples studied in this thesis. Analytical-grade reagents used throughout the study and their specifications are listed in table 3.

Table 2: Summary of investigated samples

No	Name	Date	Location
1	Old shoots	7.7.2025	Stallogargo
2	Young shoots	7.7.2025	Stallogargo
3	Old shoots	4.7.2025	Kvalsunddalen
4	Young shoots	4.7.2025	Kvalsunddalen
5	Reindeer faeces	7.7.2025	Stallogargo
6	Litters	4.7.2025	Kvalsunddalen

Table 3: List of chemicals used and their specifications

Chemical	Purity	CAT number	Provider
Methanol	>99.9%	20864.320	VWR
Batatasin-III	99.16%	TN1433	TargetMol
1,6 - dihydroxynaphthalene	>99%	D0591	TCI
Pyridine	99.8%	270970	Sigma-Aldrich
Methoxyamine hydrochloride	98%	226904	Sigma-Aldrich
n,o-bis(trimethylsilyl) trifluoroacetamide (BSTFA)	>99%	15222	Sigma-Aldrich
Trimethylsilyl chloride (TMCS)	>99%	89595	Sigma-Aldrich
Gallic acid	>98%	8.42649.0025	Merck
Folin & Ciocalteu's phenol reagent	Analytical-grade	F9252	Sigma-Aldrich
Sodium carbonate	99.95-100.05%	033377.36	Thermo Scientific
Rutin hydrate	>94%	R5143	Merck
Aluminium chloride	>99%	06232	Honeywell
Sodium nitrite	>99%	1.06549.0100	Merck
Sodium hydroxide	>97%	28240.361	VWR

3.2 Methods

This sub-sector provides detailed information about the research procedure. First, the plant matrix and reindeer faeces will be pre-treated before extraction. Single-factor experiments are conducted to pre-screen the appropriate range for the investigation parameters. A Box-Behnken design is then applied to find the optimum point for recovering phenolics and flavonoids. The experimental results from this optimization procedure will be evaluated and

compared using TPC and TFC. Next, the extracts will be derivatized and analysed using GC-MS. The profiling of both matrices will be studied and discussed. Figure 1 shows the workflow of the thesis.

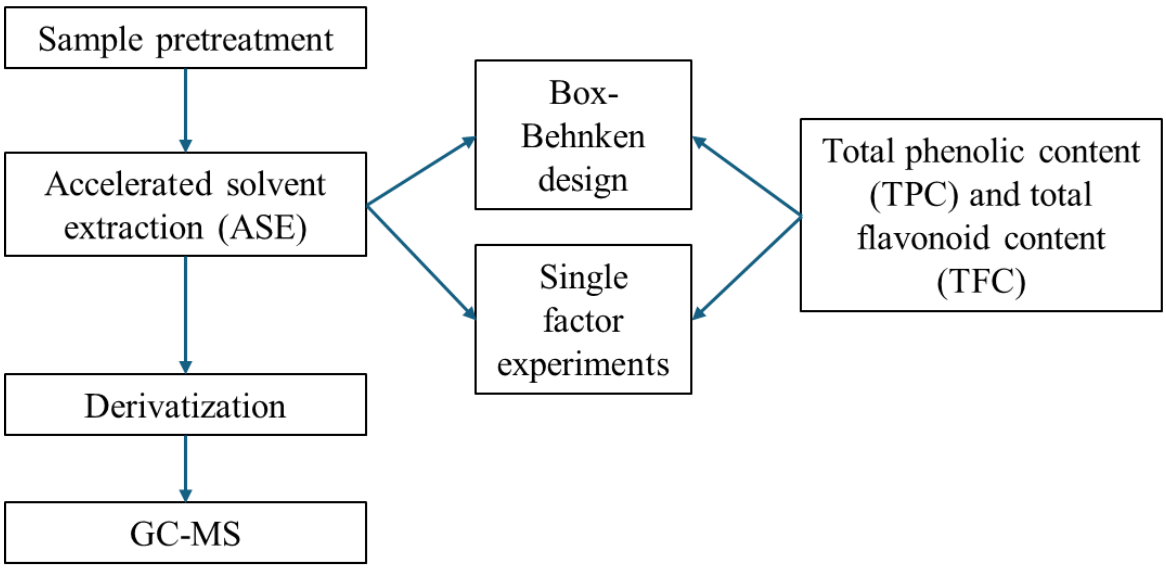


Figure 1: Diagram of workflow

3.2.1 Sample preparation

Plant and faeces samples were freeze-dried before the extraction process to reduce moisture content and improve the reproducibility of the method. Pre- and post-drying weights were recorded. The dried samples were then ground using a mechanical grinder to increase the surface area and enhance solvent penetration during extraction.

Due to sample volume limitations, a mixture of three samples, young shoots, old shoots, and litter from the plant matrix, was used to optimize the extraction process. This is because all three samples are above-ground parts of *Empetrum nigrum*, thus possessing similar characteristics to each other and to the samples to be analysed. The sample mixture was thoroughly ground using a grinder to ensure homogeneity between extractions. For the reindeer faces matrix, the sample was kept intact, and pretreatment was performed.

Regarding the extraction and analysis process after ASE optimization, the sample is divided in a different way to capture the characteristic features of each different matrix. Two samples of young shoots collected from the two mentioned locations were mixed together. The same procedure was also applied to old shoots. These samples were extracted, and their profiles were discovered separately after the ASE optimization. The same sampling method was also applied to determine the batatatsin-III content in each matrix: young shoots, old shoots, litters, and reindeer faeces.

3.2.2 Accelerated solvent extraction (ASE)

ASE was used as the primary extraction method for recovering phenolic and flavonoid compounds from both matrices. The method was altered from the study of Wang et al (2022). The extraction process was optimized based on three main parameters: methanol concentration, extraction temperature, and extraction time. First, 1 g of dry sample was mixed with diatomaceous earth at a ratio of 1:2 (w/w) and transferred into an ASE extraction cell. The solid mixture was surrounded by approximately 5 g of diatomaceous earth distributed in two layers, upper and lower, to fix the sample in the cell. Cellulose membranes were utilized to prevent small particles from entering the extracts, placed at both the top and bottom of the cell.

ASE system was operated at a constant pressure of 1500 psi with a 40% washing volume and a nitrogen purging time of 100 seconds. All experiments were performed in three cycles. After extraction, the rotary evaporator and nitrogen evaporation were used to dry the extracts completely. The residues were then redissolved in exactly 2 mL of methanol before further analysis.

3.2.3 Experimental design

First, preliminary single-factor experiments were conducted to assess the influence of extraction conditions on the phenolic and flavonoid recovery efficiency of the method. Methanol concentrations were studied at four levels (40%, 60%, 80%, and 100%), extraction temperatures at five levels (40, 60, 80, 100, and 120°C), and extraction times at three levels

(5, 10, and 15 minutes). The results of these experiments were used to narrow down the range of suitable parameters for the subsequent optimization design. Table 4 summarizes the single-factor experiments performed in this research.

Table 4: Single factor experiments design

No	Solvent concentration (%)	Temperature (°C)	Time (minutes)
1	40	80	10
2	60	80	10
3	80	80	10
4	100	80	10
5	80	40	10
6	80	60	10
7	80	80	10
8	80	100	10
9	80	120	10
10	80	80	5
11	80	80	10
12	80	80	15

Following preliminary optimization, response surface methodology (RSM) was utilized to investigate ASE operational parameters based on the Box–Behnken design (BBD). Methanol concentration, extraction temperature, and extraction time were studied at three levels (-1, 0, 1). The experimental design makes it up to 15 experimental runs. Results were used to evaluate linear, quadratic effects, and interactions between extraction variables. Table 5 shows the optimized experimental design for the ASE extraction method for both matrices based on the ranges selected from single-factor experiments.

TPC and TFC are criteria for evaluating the extraction efficiency of each experiment as described in sections 3.2.4 and 3.2.5.

Table 5: Box-Behnken experimental design

No	Solvent concentration	Temperature	Time
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

3.2.4 Total phenolic content (TPC) determination

Folin–Ciocalteu method, according to Schneider et al. (2023), with minor modifications, was utilized to determine TPC. A reference standard for calibration was gallic acid. All the samples and standards were measured at 765 nm via UV–Vis instrument.

Before analysis, the *Empetrum nigrum* extract was diluted 100 times, while the reindeer faeces extract was diluted 50 times. Then, prepare the Folin-Ciocalteu reagent at a concentration of tenfold diluted with ultrapure water. A diluted sample volume of 0.5 mL was taken and introduced to 2.5 mL of the previously prepared reagent solution. After 5 minutes, 7.5% sodium carbonate was pipetted with the volume of 2 mL into the solution. The mixture was stirred for 10-15 seconds.

Incubation was performed in darkness at around 25ºC for 2 hours to allow the colour complex to develop. All analytical experiments were conducted three times. The obtained absorbance values were used to calculate the TPC based on the constructed calibration curve. The unit is milligrams of gallic acid equivalent (GAE) per gram of dry weight for both *Empetrum nigrum* and reindeer faeces samples. The TPC of the *Empetrum* plant matrix was

determined using equation (2), while that of the reindeer faeces sample was presented in equation (3).

$$TPC_{plant} = \frac{C_{phenolic-plant} \times 100 \times V}{m} \quad (2)$$

With TPC_{plant} is total phenolic content of plant matrix (mg GAE/ g DW), $C_{phenolic-plant}$ is concentration of phenolic compounds calculated by the standard curve (mg/L), V is volume of extract (L), m is the weight of the dried plant (g).

$$TPC_{reindeer} = \frac{C_{phenolic-reindeer} \times 50 \times V}{m} \quad (3)$$

With $TPC_{reindeer}$ is total phenolic content of reindeer faeces matrix (mg GAE/ g DW), $C_{phenolic-reindeer}$ is concentration of phenolic compounds calculated by the standard curve (mg/L), V is volume of extract (L), m is the weight of the dried plant (g).

The calibration curve is constructed based on different gallic acid concentrations in the range 0 - 500 mg/L. The blank solution consists of distilled water and reagents.

3.2.5 Total flavonoid content (TFC) determination

The colorimetric method using aluminum chloride reagent according to Zhang and Tai (2021) was employed to determine TFC. Rutin was used as a reference standard. All samples and standards were analyzed at a wavelength of 510 nm using a UV-Vis device.

Before performing the analysis with the reagent, the *Empetrum* extract was diluted 100 times, and 50 times for the reindeer faeces matrix. First, the test tubes were fill with 2.5 mL of ultra-pure water and add, respectively 0.5 mL of the diluted sample extract and 150 µL of 5% sodium nitrite solution. The incubation process lasted for five minutes, and 150 µL of 10% aluminum chloride solution was poured in to activate the mixture. The reaction mixture was stood still for approximately 6 minutes before adding 1 mL of sodium hydroxide. The solution was then stirred thoroughly with a vortex stirrer for about 10-15 seconds, and left

in the dark for 40 minutes. When the colour was stable, UV-Vis was used to analyse the samples.

All measurements were performed three times. The rutin calibration curve was a database used to determine TFC, and results unit is in milligrams of rutin equivalent (RE) per gram of dry weight for both *Empetrum nigrum* and reindeer faeces samples. The TFC of the *Empetrum* plant matrix was evaluated using equation (4), while that of the reindeer faeces sample was presented in equation (5).

$$TFC_{plant} = \frac{C_{flavonoid-plant} \times 100 \times V}{m} \quad (4)$$

With TFC_{plant} is total flavonoid content of plant matrix (mg RE/ g DW), $C_{flavonoid\text{-}reindeer}$ is concentration of flavonoid compounds calculated by the standard curve (mg/L), V is volume of extract (L), m is the weight of the dried plant (g).

$$TFC_{reindeer} = \frac{C_{flavonoid-reindeer} \times 50 \times V}{m} \quad (5)$$

With $TFC_{reindeer}$ is total flavonoid content of plant matrix (mg RE/ g DW), $C_{flavonoid\text{-}reindeer}$ is concentration of flavonoid compounds calculated by the standard curve (mg/L), V is volume of extract (L), m is the weight of the dried plant (g).

Different rutin concentrations ranging from 0-500 mg/L were selected to construct the calibration curve. Blank solution consisted of methanol and reagents.

3.2.6 Gas chromatography – Mass spectroscopy (GC-MS)

- Derivatization:

Before the samples were analysed by GC-MS, a derivatization procedure was performed. The steps below have been adapted from the publication by Muravnik et al (2012). 20µL of the crude extract was placed in a GC vial and evaporated with nitrogen until dry. The dry

extract sample was dissolved in 50 µL of pyridine containing methoxyamine hydrochloride with the concentration of 20 mg/mL. Methoxyamine hydrochloride acts as a stabilizer for carbonyl groups such as sugars, aldehydes, and ketones. The vial was then left incubated at 60°C for 60 minutes and further evaporated until completely dry. The residue was redissolved in 50µL of pyridine containing 10 ng/µL 1,6 - dihydroxynaphthalene as an internal standard. To initiate the derivatization reaction, 50 µL of 99% BSTFA with 1% TMCS was put into the vial. The reaction was activated when the temperature was heated up to 100°C for 30 minutes to facilitate the formation of trimethylsilyl derivatives.

After derivatization, the prepared samples were directly analysed by GC-MS.

- Analytical procedure:

To quantify batatasin-III, a silica DB-5 capillary column is used. The column dimension was 30 m x 0.25 mm ID, and 0.25 µm was the film thickness. 1 µL was selected as the injection volume. 1 ml/min is represented for the helium gas flow rate. Initial temperature is set from 70°C to 320°C with a steady growth of 5°C per minute. Batatasin-III concentration is estimated by the internal standard methodology based on the batatasin-III and 1,6-dihydroxynaphthalene (internal standard) area ratio plot with batatasin-III concentration range from 5-300 ng/µL

Batatasin-III in the matrix is measured by mass spectrometry coupled with gas chromatography. The parameters are adjusted similarly to those in the gas chromatography analysis mentioned above. 70 eV is set for the ionization energy to obtain electron collision mass spectra with an ion source temperature of 250°C. 50–600 is a mass range to record the total ion current. (Wallstedt et al 1997)

Upon receiving the analysis results, batatasin-III in the reindeer faeces sample was detected at a low level. Therefore, to obtain more accurate results, selected ion monitoring (SIM) mode was applied. Operational parameters were similar to those described above. For internal standard 1,6 - dihyroxynaphthalene, the quantitative ion used was m/z 209 and the qualitative ion was m/z 304, while for the target compound batatasin-III, the quantitative ion used was m/z 289 and the qualitative ion was m/z 388.

4 Results and discussion

4.1 Sample pretreatment

Table 6 presents the preliminary processing results of the collected samples, including old shoots, young shoots, leaf litter, and reindeer faeces. The table 6 summarizes the sampling location, collection date, sample weight before and after freeze-drying, and calculated percentage of water content. Freeze-drying was performed to remove moisture from the samples before extraction, thereby improving sample stability, preventing microbial degradation, and allowing for more accurate comparison of extraction efficiency on a dry weight basis.

The results showed significant differences in water content between the different sample types. Young shoots had the highest water content, reaching 72.18% and 65.59%, respectively. The above observation is reasonable with the physiological characteristics of young plant tissue, which typically contains higher water content due to growth and active metabolic activity. Conversely, older shoots had lower water content, around 45–47%. Meanwhile, the litter samples showed an average water content of 56.5%, which may reflect partial decomposition and exposure to the environment before collection.

The reindeer faeces samples had a water content of 49.05%, similar to that observed in samples of old plant shoots. This could be due to the relatively long harvesting time and prolonged freezing. Furthermore, the preliminary imaging of the reindeer faeces samples is complex, containing heterogeneous components such as undigested plant matter, and biomass.

Table 6: Sample pretreatment results

No	Name	Date	Location	Mass before freeze-drying (g)	Mass after freeze-drying (g)	Water content (%)
1	Old shoots	7.7.2025	Stallogargo	23.92	12.68	46.99
2	Young shoots	7.7.2025	Stallogargo	8.52	2.37	72.18
3	Old shoots	4.7.2025	Kvalsunddalen	17.55	9.71	44.67
4	Young shoots	4.7.2025	Kvalsunddalen	5.58	1.92	65.59
5	Litters	4.7.2025	Kvalsunddalen	35.62	17.77	56.50
6	Reindeer faeces	7.7.2025	Stallogargo	142.22	74.96	49.05

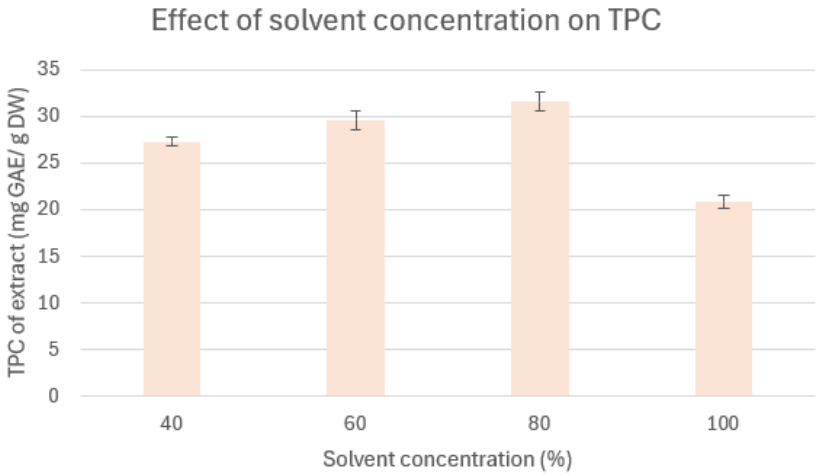
4.2 Single-factor experiments

4.2.1 Plant matrix

Single-factor experiments were conducted by changing one variable while keeping the others constant. Results from these experiments were rated using total phenolic content (TPC) and total flavonoid content (TFC). $y = 0.0048x + 0.1307$ with $R^2 = 0.9938$ represents the calibration curve of TPC (Appendix). The standard curve for TFC was applied as $y = 0.0014x + 0.0173$ with $R^2 = 0.997$ (Appendix).

Figure 2 summarizes the TPC investigation results of the *Empetrum* plant samples. Methanol solvent concentration was studied at four levels, and the highest TPC was achieved at 80% methanol with regarding value at 31.60 ± 0.97 mg GAE/g DW. A resembling pattern was witnessed under operating temperature conditions. Temperatures were investigated from 40 to 120°C. The highest TPC reached 37.45 ± 0.70 mg GAE/g DW at 100ºC. Meanwhile, no notable difference was witnessed between the extraction times of 10 and 15 minutes, with TPC values respectively of approximately 35.68 ± 0.45 mg GAE/g DW and 35.70 ± 0.18 mg GAE/g DW.

In summary, preliminary experiments show that the optimum operating extraction point for accelerated solvent extraction (ASE) to yield the highest TPC is a methanol concentration at 80%, an operational temperature of 100°C, and a time of 10 minutes.



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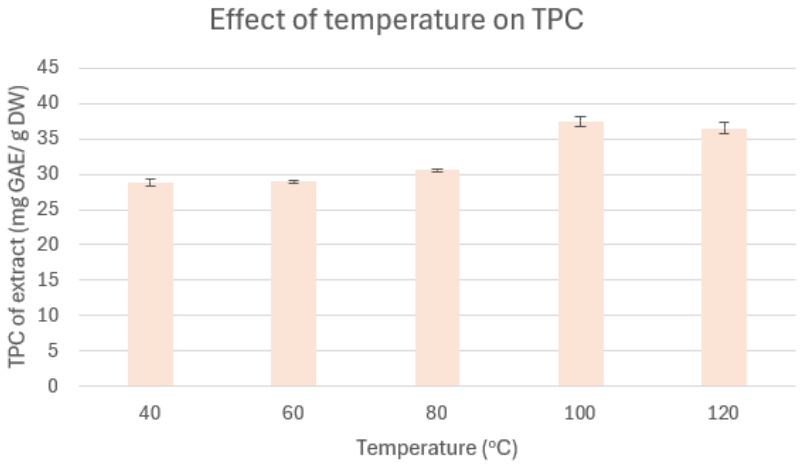

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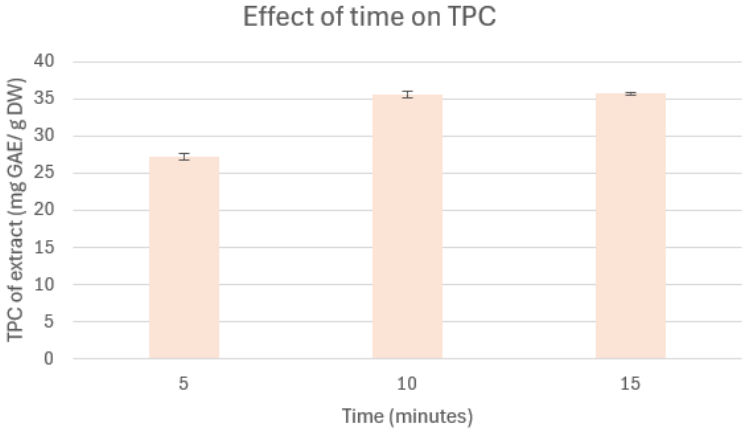
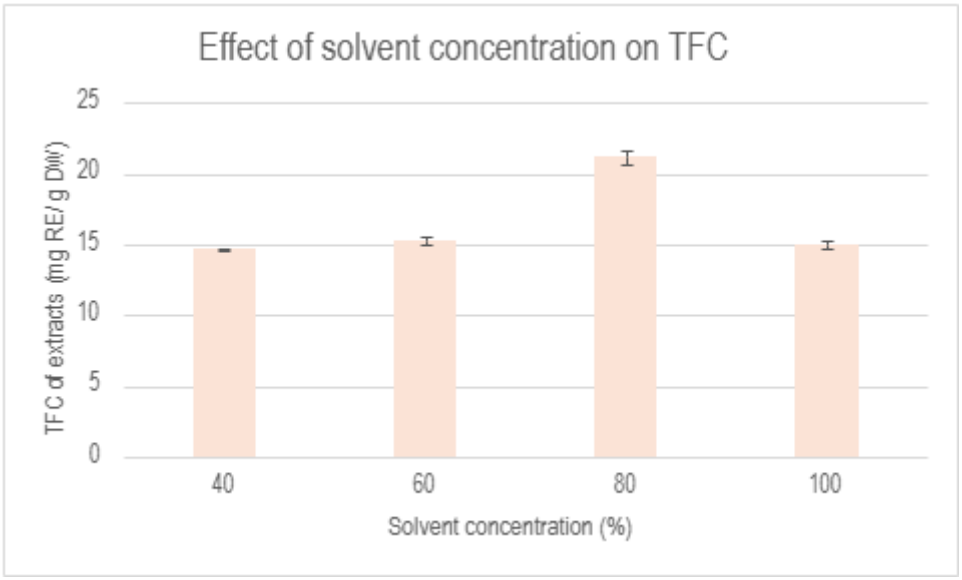


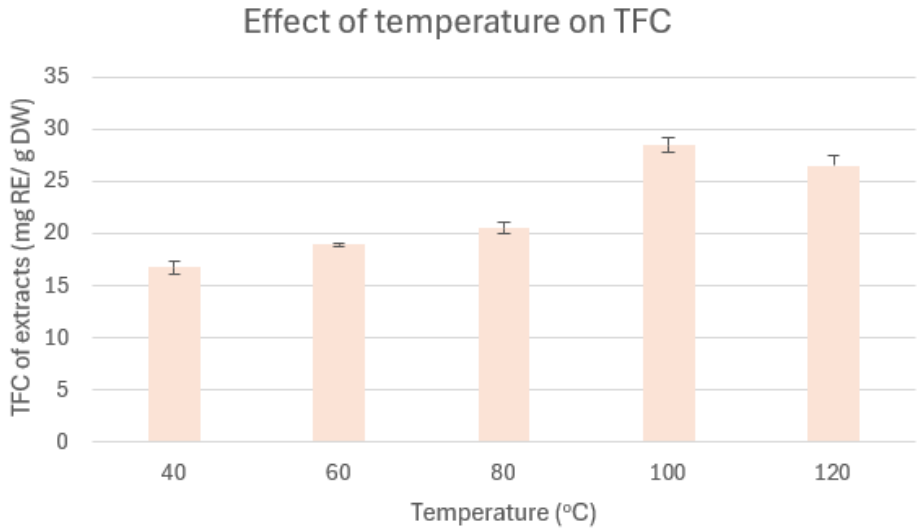
(C)

Figure 2: Investigated condition effects on TPC of plant matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

The results of the impact of solvent concentration, temperature, and extraction time on TFC of *Empetrum* sample were presented in figure 3. For the first two mentioned variables, a similar trend was observed compared to TPC. TFC reached its highest value at a methanol concentration of 80% and a temperature of 100°C, with the corresponding values of 21.18 ± 0.53 mg RE/g DW and 28.44 ± 0.68 mg RE/g DW. Conversely, longer extraction times resulted in higher TFC values between 10 and 15 minutes, although the difference was insignificant.



(A)



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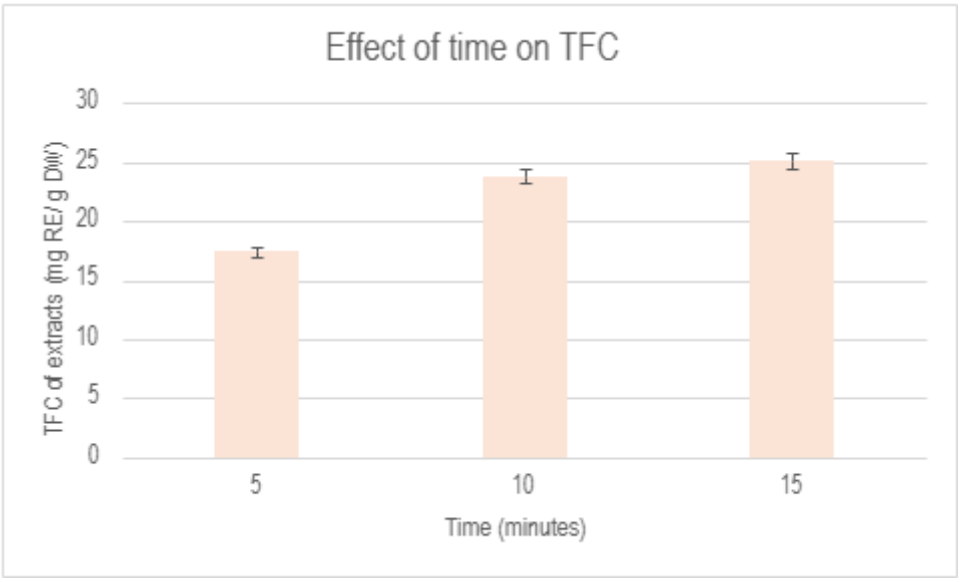


Figure 3: Investigated condition effects on TFC of plant matrix (A) solvent concentration, (B) Temperature, (C) Time, and error bars are reported as mean ± SD (n = 3).

Table 7 summarized TPC and TFC of plant matrix under various extraction conditions, expressed as mean ± standard deviation (SD).

Table 7: TPC and TFC of plant matrix under investigated conditions

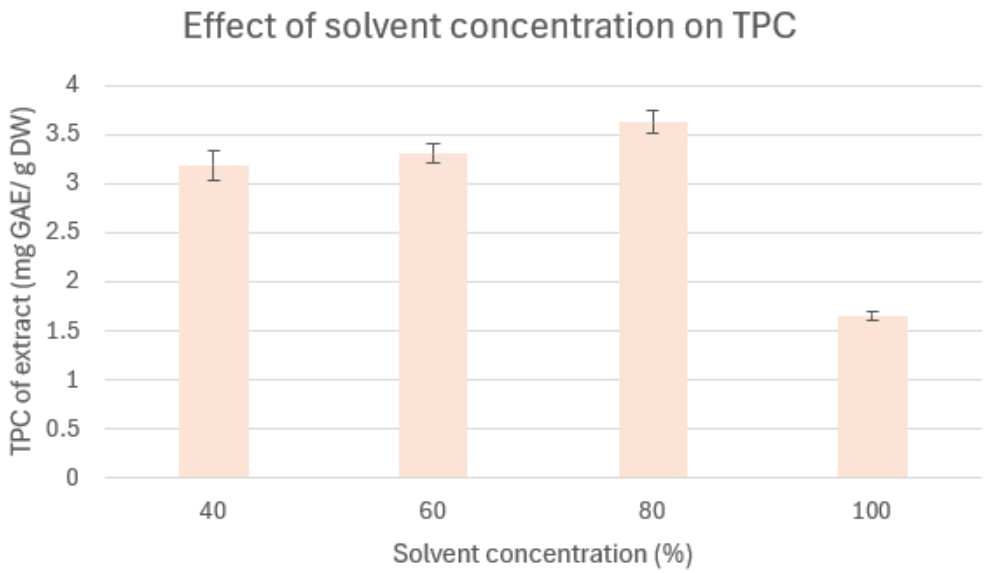
Factors	Levels	Mean of TPC (mg GAE / g DW)	SD (n=3)	Mean of TFC (mg RE / g DW)	SD (n=3)
Solvent concentration (%)	40	27.30	0.48	14.68	0.05
	60	29.60	0.97	15.23	0.28
	80	31.60	0.97	21.18	0.53
	100	20.80	0.70	14.98	0.32
Temperature (°C)	40	28.86	0.51	16.74	0.64
	60	28.93	0.16	18.96	0.16
	80	30.53	0.23	20.54	0.47
	100	37.45	0.70	28.44	0.68
	120	36.50	0.82	26.52	0.91
Time (minutes)	5	27.19	0.42	17.49	0.43
	10	35.68	0.45	23.86	0.57
	15	35.70	0.18	25.14	0.63

The selection of extraction conditions for the *Empetrum* sample must be balanced to achieve high TPC and TFC levels. 80% methanol, a working temperature of 100°C, and an extraction time of 10 minutes were considered to be the optimal values for further research.

4.2.2 Reindeer faeces matrix

The influence of extraction condition variables on the TPC of the reindeer faeces matrix is shown in Figure 4. There is a clear difference between TPC at 80% methanol and 100% methanol levels, 3.63 ± 0.11 mg GAE/g DW compared to 1.66 ± 0.05 mg GAE/g DW. Furthermore, an interesting data point was observed at the extraction temperature condition as TPC gradually increased to the highest temperature in the study at 120°C and could reach even higher values with further temperature increases. This suggests that a local optimal point for TFC has not yet been found for the temperature parameter. Meanwhile, TPC in the reindeer faeces matrix also did not differ significantly between the two extraction time points of 10 and 15 minutes, with corresponding values of 3.16 ± 0.10 mg GAE/g DW and 3.05 ± 0.12 mg GAE/g DW.

Thus, for TPC, the optimal extraction conditions for this matrix are 80% methanol, a temperature of 120°C, and a period of time within approximately 10 minutes.



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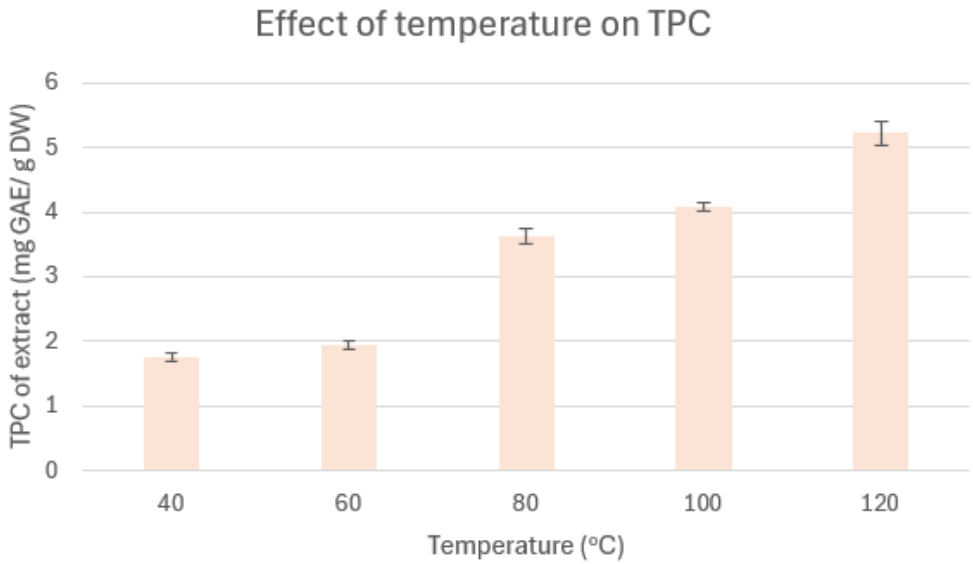

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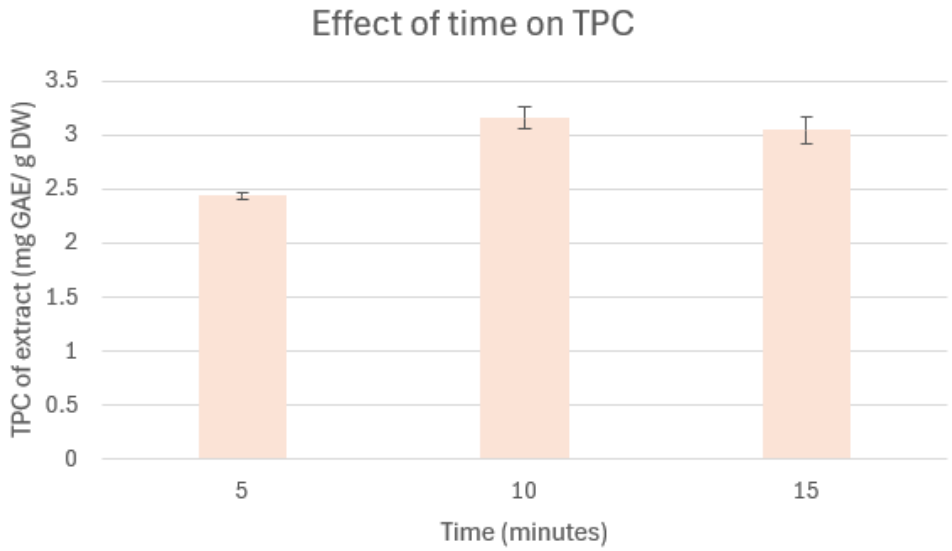
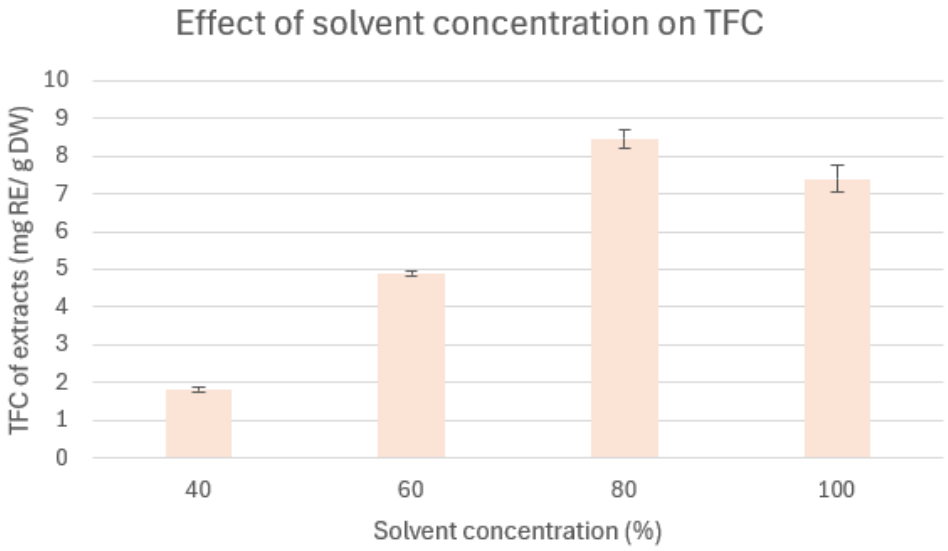


(C)

Figure 4: Investigated condition effects on TPC of reindeer faeces matrix (A) solvent concentration, (B) Temperature, (C) Time and error bars are reported as mean ± SD (n = 3).

A similar pattern was found in the TFC value when compared to TPC in Figure 5. TFC reached its highest value at 8.46 ± 0.24 mg RE/g DW when the solvent concentration was 80%. At 120°C, TFC was observed and predicted to continue increasing further when the temperature increased. The optimal extraction time for TFC was 10 minutes, with a slight decrease in TFC value with longer extraction times (15 minutes).

For the reindeer faeces matrix, the optimal extraction parameters were set at 80% methanol, temperature at 120°C, and time of 10 minutes.



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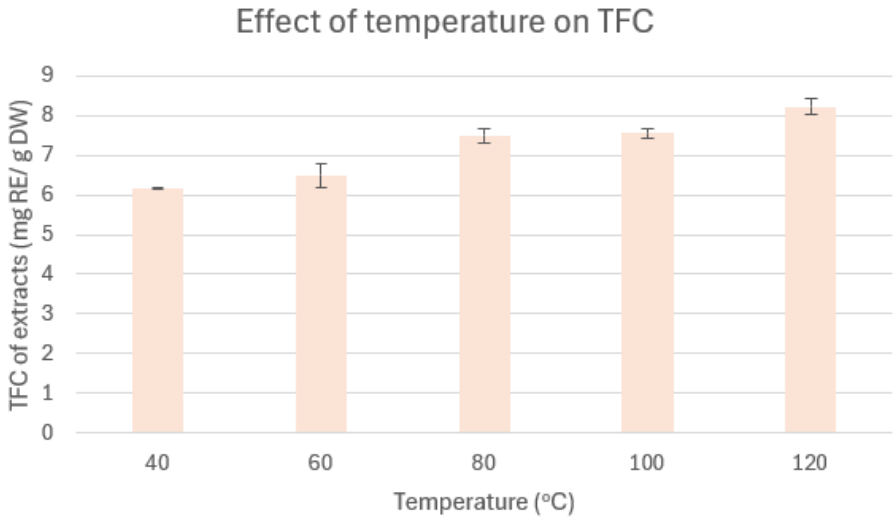

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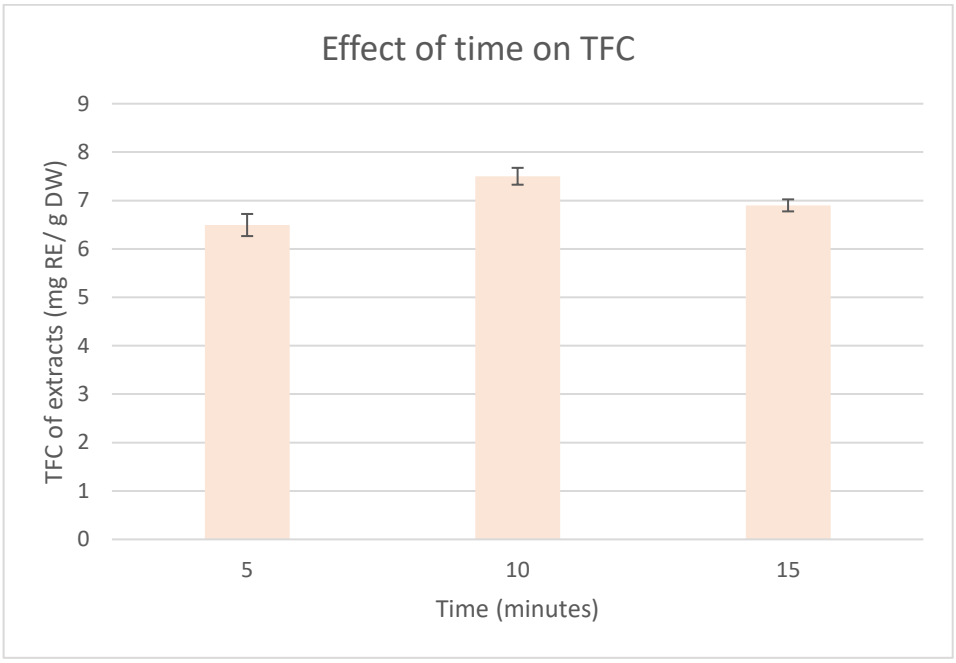
(C) 

Figure 5: Investigated condition effects on TFC of reindeer faeces matrix (A) solvent concentration, (B) Temperature, (C) Time and error bars are reported as mean ± SD (n = 3).

Table 8 showed the deviation of TPC and TFC of reindeer faeces matrix under different extraction conditions. The above results, in both plant and faecal matrices, will be applied as the central point in a further optimization study applying the RSM.

Table 8: TPC and TFC of reindeer faeces matrix under investigated conditions

Factors	Levels	Mean of TPC (mg GAE / g DW)	SD (n=3)	Mean of TFC (mg RE / g DW)	SD (n=3)
Solvent concentration (%)	40	3.19	0.15	1.81	0.06
	60	3.30	0.10	4.88	0.05
	80	3.63	0.11	8.46	0.24
	100	1.66	0.05	7.40	0.36
Temperature (°C)	40	1.77	0.07	6.16	0.02
	60	1.94	0.07	6.49	0.32
	80	3.63	0.11	7.50	0.17
	100	4.09	0.07	7.56	0.12
	120	5.22	0.19	8.22	0.20
Time (minutes)	5	2.44	0.04	6.49	0.23
	10	3.16	0.10	7.50	0.17
	15	3.05	0.12	6.90	0.12

4.3 Box-Behnken design

4.3.1 Plant matrix

According to the extraction outcomes from single-factor experiments, the optimization procedure was designed at three levels. For the *Empetrum nigrum* matrix, the Box-Behnken design (BBD) was applied as described in table 5 with solvent concentrations of 60% methanol (level -1), 80% methanol (level 0), and 100% methanol (level 1). Extraction temperatures were 80°C (level -1), 100°C (level 0), and 120°C (level 1), while extraction times were 5, 10, and 15 minutes, corresponding to the three levels mentioned above, respectively. Table 9 summarized experimental run for *Empetrum nigrum* samples.

Table 9: Box-Behnken design for *Empetrum nigrum* matrix

No	Solvent concentration (%)	Temperature (ºC)	Time (minutes)
1	60 (-1)	80 (-1)	10 (0)
2	60 (-1)	120 (1)	10 (0)
3	100 (1)	80 (-1)	10 (0)
4	100 (1	120 (1)	10 (0)
5	80 (0)	100 (0)	5 (-1)
6	80 (0)	100 (0)	5 (-1)
7	80 (0)	100 (0)	15 (1)
8	80 (0)	100 (0)	15 (1)
9	60 (-1)	80 (-1)	5 (-1)
10	100 (1	80 (-1)	5 (-1)
11	60 (-1)	120 (1)	15 (1)
12	100 (1	120 (1)	15 (1)
13	80 (0)	100 (0)	10 (0)
14	80 (0)	100 (0)	10 (0)
15	80 (0)	100 (0)	10 (0)

After extraction with ASE, the extracted solution was observed to be brownish and separated if left to settle for a long time. This is predicted to be caused by phenolic compounds and plant cell debris that passed through during the extraction process, which is reasonable given that crowberries are famous for their high and abundant amounts of phenolic compounds. Figure 6 shows the colour and matrix texture of the extracted solution throughout the 15 experimental runs.

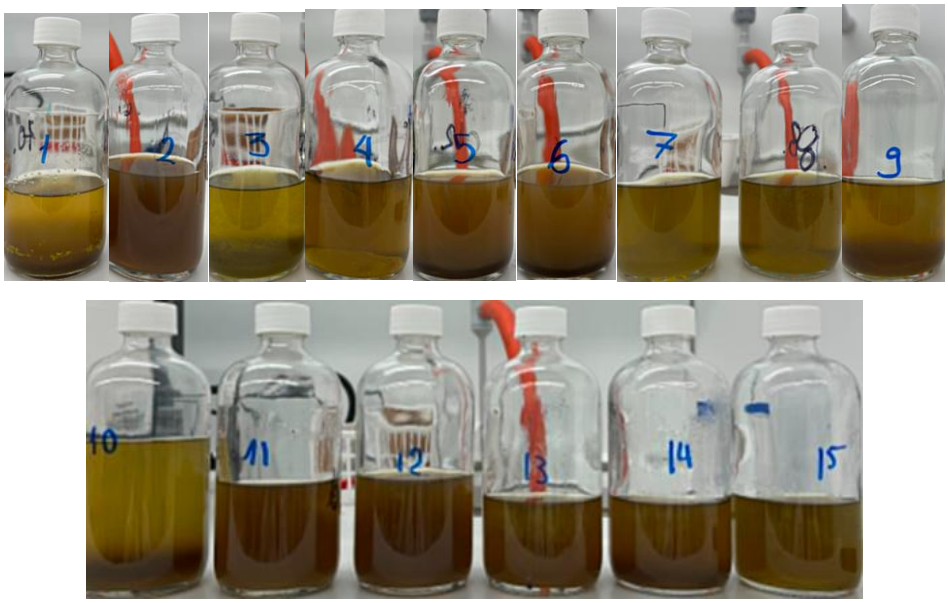


Figure 6: *Empetrum* extracts throughout 15 experimental extractions

Similar to single-factor experiments, extraction conditions will be optimized based on the TPC and TFC of the investigated samples. Table 10 shows the experimental outcomes based on the Box-Behnken model.

Table 10: ANOVA summary table according to TPC and TFC responses of *Empetrum* matrix with coefficients of model equation, sum of squares (SS), degrees of freedom (DF), mean squares (MS), F statistic (F-value), and probability value (p-value)

	TPC					
Factor	Coefficient	SS	DF	MS	F-value	p-value
Model		1067.5200	9	118.6130	30,5994	0.0008
Linear term		754.9600	3	251.6520	64,9205	0.0002
x_1	-9.6162		1		-13.8146	3.569e-05
x_2	0.2124		1		0.3052	0.7725
x_3	1.3613		1		1.9556	0.1079
Quadratic		289.0100	3	96.3370	24.8527	0.0002
x_1^2	-8.3879		1		-8.1864	0.0004
x_2^2	-3.4225		1		-3.3403	0.0205
x_3^2	-0.4497		1		-0.4389	0.6791
Interactions		23.5500	3	7.8500	2.025	0.2291
x_1x_2	1.6442		1		1.6702	0.1557
x_1x_3	1.3610		1		1.3826	0.2254
x_2x_3	1.1539		1		1.1721	0.2940
Lack of fit		16.6100	3	5.5360	3.9944	0.2067
Pure error		2.7700	2	1.3860		
R^2		0.9822				

	TFC					
Factor	Coefficient	SS	DF	MS	F-value	p-value
Model		368.5590	9	40.9510	5.4678	0.0380
Linear term		154.6420	3	51.5470	6.8826	0.0317
x_1	-4.0754		1		-4.2120	0.0084
x_2	0.5177		1		0.5351	0.6155
x_3	1.5663		1		1.6188	0.1664
Quadratic		202.7930	3	67.5980	9.0257	0.0184
x_1^2	-6.4553		1		-4.5325	0.0062
x_2^2	-4.0626		1		-2.8525	0.0357
x_3^2	-1.4256		1		-1.0009	0.3628
Interactions		11.1240	3	3.7080	0.4951	0.7013
x_1x_2	1.2818		1		0.9367	0.3919
x_1x_3	0.4938		1		0.3609	0.7329
x_2x_3	0.9456		1		0.6910	0.5203
Lack of fit		23.9670	3	7.9890	1.1853	0.4880
Pure error		13.4800	2	6.7400		
R^2		0.9078				

The effect of solvent concentration (x_1), extraction temperature (x_2), and extraction time (x_3) on phenolic recovery efficiency by ASE was evaluated by response surface methodology (RSM). The statistical results were demonstrated by the variance analysis (ANOVA) where the model's p-value was quite small (0.0008), demonstrating that the selected variables explained the variation in the extraction process. In addition, the coefficient of determination R^2 value of 0.9822 was, meaning that the model explained 98.2% of the observed variation in TPC among the extraction conditions. With p-value = 0.2067 > 0.05, the lack of fit is considered negligible, confirming the reliability and accuracy of the model, and the quadratic equation (6) could predict the TPC response to find the optimal point.

$$TPC = 77.0646 - 8.3879x_1^2 - 3.4225x_2^2 - 0.4497x_3^2 - 9.6162x_1 + 0.2124x_2 + 1.3613x_3 + 1.6442x_1x_2 + 1.3610x_1x_3 + 1.1539x_2x_3 \quad (6)$$

Among the linear terms, solvent concentration (x_1) had the most significant effect on extraction results, with a negative coefficient (-9.6162). This number indicates that TPC concentration would increase sharply when using the solvent at lower concentrations. This strong effect is consistent with previous reports that solvent polarity is one of the crucial aspects in the extraction process (Wang et al 2021). Typically, bioactive compounds will have different polarities, so optimizing the process with different solvent concentrations will yield suitable phenolic recovery results. Mixing water and solvent will adjust the polarity of the solution, facilitating solvent penetration into plant tissue and improving the mass transfer of the phenolics (Dai and Mumper 2010). If the solvent concentration is too high, it can reduce extraction efficiency by disrupting the polarity balance necessary to dissolve a range of phenolic components.

In contrast, extraction temperature and time factors showed negligible effects on TPC values, with the coefficients being much smaller than those of solvent concentration (x_1). This explains why increasing the temperature and extraction time only slightly altered the phenolics extraction yield. Furthermore, the extraction time also showed a clear advantage when ASE conditions were used, resulting in efficient extraction in a short time, under 15 minutes.

The quadratic terms provided a more comprehensive view of the extraction behavior. Both the quadratic effects of solvent concentration (x_1^2, $p < 0.001$) and temperature (x_2^2, $p = 0.021$) were statistically significant and negative. This means that the curvature of the model surface explained the optimization of extraction conditions within the experimental range. The negative quadratic coefficients indicate that excessively low or high solvent concentrations would reduce TPC recovery efficiency. This further confirms the importance of the polarity of the extraction solvent in maximizing the solubility of various phenolic compounds. Similarly, increasing the operational temperature increased the extraction efficiency; however, excessively high temperatures can cause degradation of natural compounds.

Furthermore, the interaction effect coefficients x_1x_2, x_1x_3, and x_2x_3 are quite small, which indicates minimal variation in the model. This suggests that the main factors influencing the TPC extraction process are more independent than synergistic within the experimentally studied scope. Figure 7 shows the interaction between experimental factors through 3D response surface contour. Each graph shows a very clear arc within the surveyed range, which proves that the model has found the true optimization point. The model successfully represents the interaction between those coefficients and found the highest TPC value within the investigated range.

Based on the equation and the response surface contour, the optimal ASE conditions for efficient TPC recovery are solvent extraction of 69.95%, operational temperature of 100.95°C, and a period of time within 14.07 minutes.

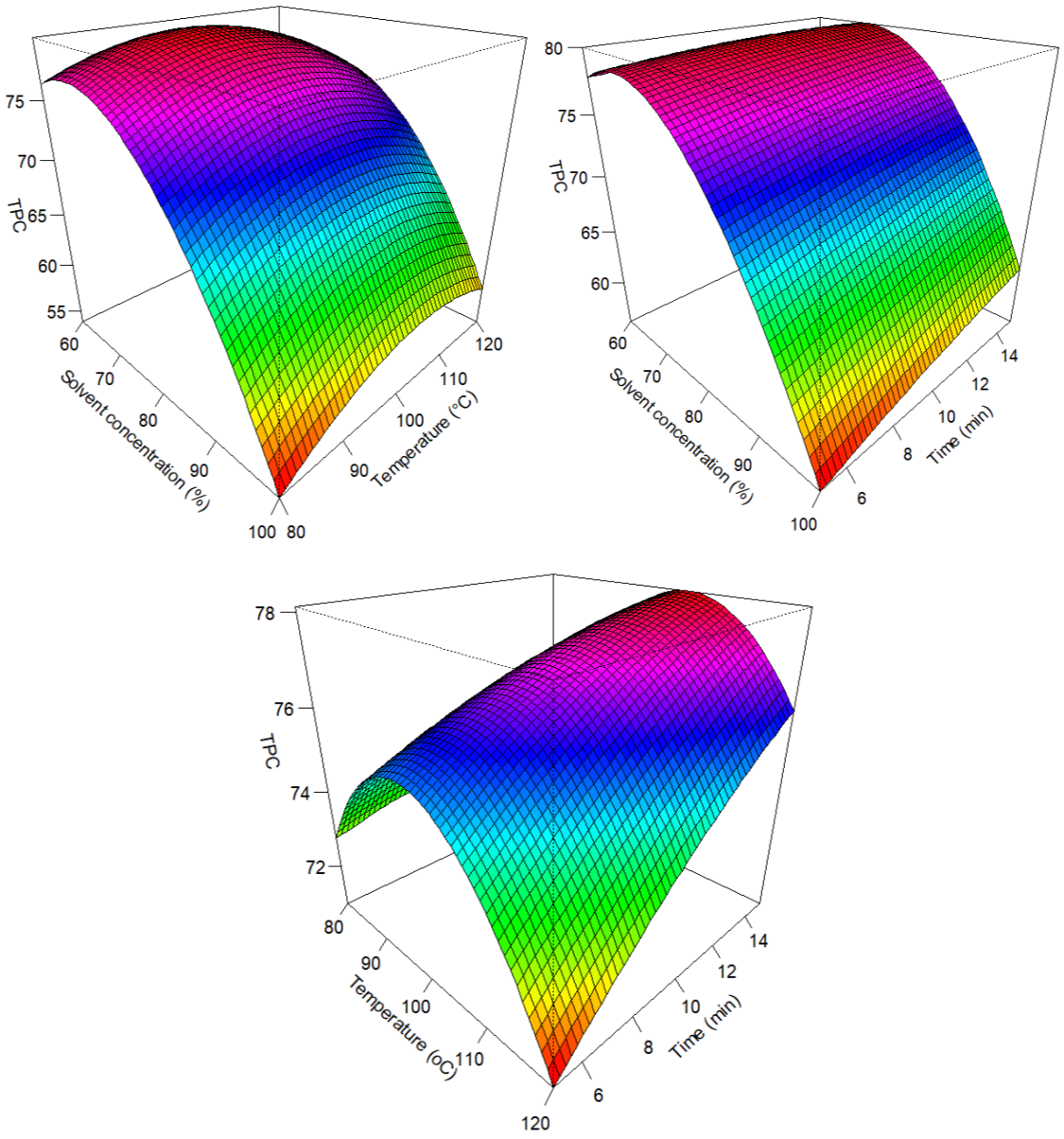


Figure 7: 3D contour of TPC response of *Empetrum* matrix

For total flavonoid content (TFC), the response surface model was effectively applied with an R^2 of 0.9078. Extraction variables were well-described regarding flavonoid recovery potential. The lack of fit was insignificant (p-value = 0.4880 > 0.005), presenting that the model could provide adequate information for the experimental data.

Similar to the TPC results, solvent concentration once again demonstrated its importance in flavonoid recovery. Significant negative coefficients at both linear (x_1) and quadratic (x_1^2) order showed that flavonoid recovery strongly depended on solvent composition, and

optimal extraction occurred at medium solvent concentrations. Thus, phenolic and flavonoid compounds tended to be more effectively dissolved and extracted in a lower solvent concentration environment. Equation (7) explains the variation of flavonoids during the optimization process.

$$TFC = 55.9649 - 6.4553x_1^2 - 4.0626x_2^2 - 1.4256x_3^2 - 4.0754x_1 + 0.5177x_2 + 1.5663x_3 + 1.2818x_1x_2 + 0.4938x_1x_3 + 0.9456x_2x_3 \quad (7)$$

The quadratic coefficient of extraction temperature showed a remarkable influence, although there was no notable linear effect. This suggests a similar pattern to phenolic recovery. Increasing the temperature may have increased the delivery of flavonoids from the sample matrix into the extract, while excessively high temperatures may have degraded or altered heat-sensitive flavonoid compounds. Similar phenomena have been reported for other green extraction techniques, where increasing the temperature improves mass transfer and solvent penetration but may reduce the stability of some flavonoid structures at higher concentrations (Wang et al 2021).

Conversely, extraction time did not significantly affect TFC recovery efficiency. The limited effect of extraction time suggests that ASE efficiently extracts flavonoids over a relatively short period due to enhanced solvent diffusion under high temperature and pressure conditions. Similarly, interactions between experimental factors were observed to be negligible and behaved largely independently within the scope of the experiment.

Overall, the flavonoid extraction process is similar to the total phenolic extraction process, highlighting the importance of solvent composition as the main factor influencing recovery efficiency. Figure 8 illustrates the curve of the model constructed based on the flavonoid recovery results of 15 optimization experiments. For TFC, the model continues to show the arc peak within the surveyed range. This demonstrates that the quadratic model and extraction range have performed well for the *Empetrum* matrix. According to the model, the optimal points were predicted with a solvent concentration of 74.25%, a temperature of 101.59°C, and an extraction time of 12.63 minutes.

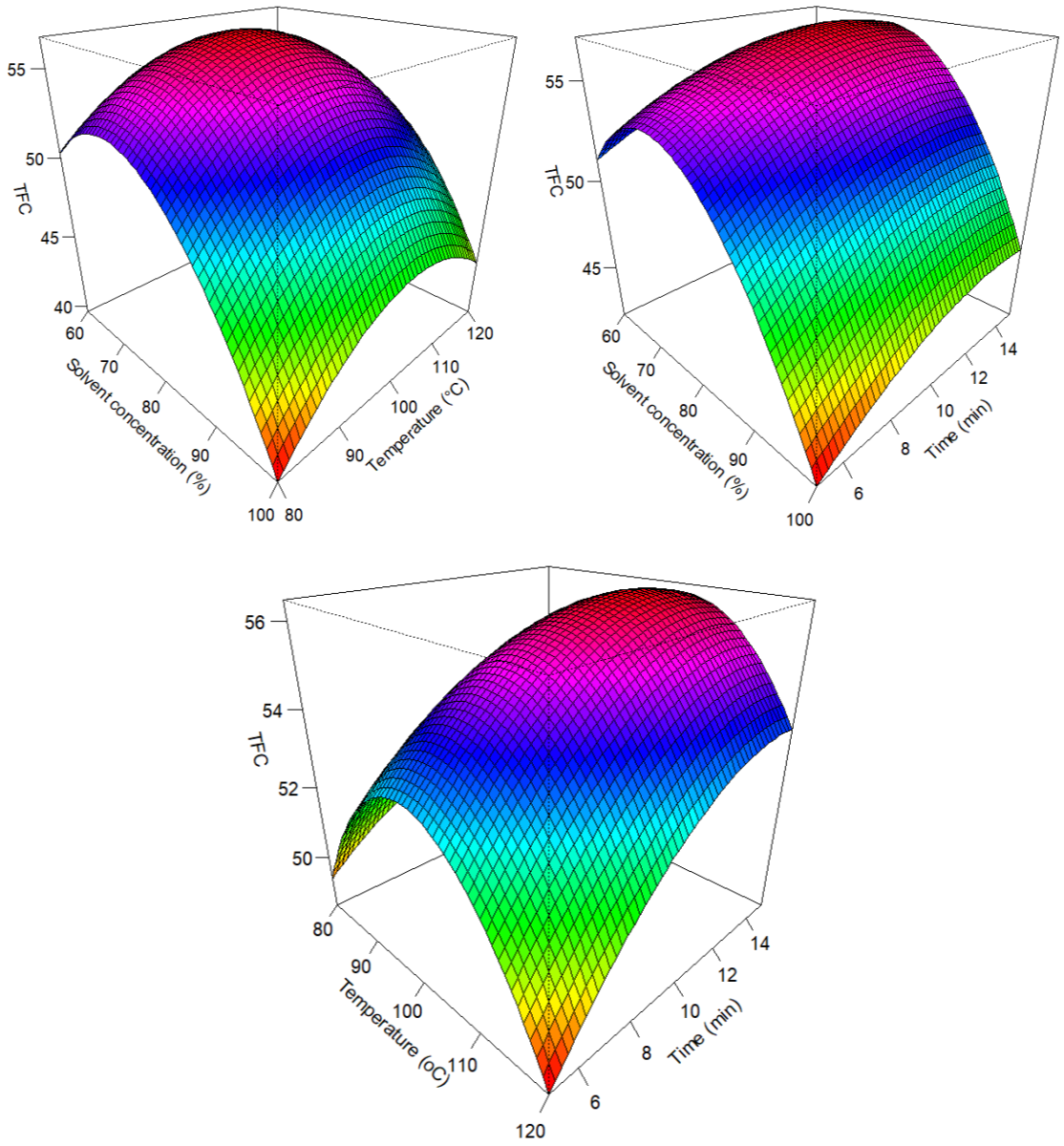


Figure 8: 3D contour of TFC response of *Empetrum* matrix

Table 11 summarizes the predicted extraction conditions based on TPC and TFC recovery efficiency. Thus, to attain the high recovery performance of both TPC and TFC for the *Empetrum nigrum* matrix, the equilibrium point was selected at a 72% methanol, and extraction temperature and time of 101°C and 13 minutes, respectively.

Table 11: Optimal extraction conditions of *Empetrum nigrum* regarding to TPC and TFC

Factor	TPC		TFC	
Methanol concentration	69.95	%	74.25	%
Temperature	100.95	ºC	101.59	ºC
Time	14.07	minutes	12.63	minutes

4.3.2 Reindeer faeces matrix

To systematize the extraction process of phenolic compounds from reindeer faeces, RSM was also applied. Table 12 represents all experimental condition optimization for reindeer faeces matrix.

Table 12: Box-Behnken design for reindeer faeces matrix

No	Solvent concentration (%)	Temperature (ºC)	Time (minutes)
1	60 (-1)	100 (-1)	10 (0)
2	60 (-1)	140 (1)	10 (0)
3	100 (1)	100 (-1)	10 (0)
4	100 (1	140 (1)	10 (0)
5	80 (0)	120 (0)	5 (-1)
6	80 (0)	120 (0)	5 (-1)
7	80 (0)	120 (0)	15 (1)
8	80 (0)	120 (0)	15 (1)
9	60 (-1)	100 (-1)	5 (-1)
10	100 (1	100 (-1)	5 (-1)
11	60 (-1)	140 (1)	15 (1)
12	100 (1	140 (1)	15 (1)
13	80 (0)	120 (0)	10 (0)
14	80 (0)	120 (0)	10 (0)
15	80 (0)	120 (0)	10 (0)

According to an R^2 value of 0.9877, it shows that nearly 99% of the observed variation in phenolic recovery efficiency can be explained by the selected extraction variables. Furthermore, p-value of 0.083 > 0.005 represented its lack of fit, demonstrating that the model can effectively described the extraction process within the investigated range. The

high predictive efficiency suggests that solvent concentration, extraction temperature, and time together played a dominant part in controlling the release of phenolic compounds from the faecal matrix. The TPC variation in the reindeer matrices is explained by equation (8).

$$TPC = 7.9310 - 1.3507x_1^2 + 1.7501x_2^2 + 1.2709x_3^2 - 3.4626x_1 + 4.0480x_2 + 1.5262x_3 - 1.6301x_1x_2 - 0.7659x_1x_3 + 0.5507x_2x_3 \quad (8)$$

Unlike the results obtained for *Empetrum nigrum*, all three extraction factors significantly affected the recovery of TPC from reindeer faeces. Solvent concentration showed a significantly negative impact, with the linearity coefficient being the most negative compared to the others. Thus, higher solvent concentrations reduced the phenolic extraction capacity. This phenomenon may reflect the heterogeneous nature of the faeces matrix, due to its complexity. In this matrix, phenolic compounds exist not only as free molecules but also as metabolites and compounds bound to partially digested plant material. Therefore, excessively high concentrations of organic solvents may reduce the extraction capacity of the more polar phenolic components present in the faeces.

In contrast, extraction temperature showed a strong positive impact on TPC recovery and was one of the most influential variables in the model, with a linearity coefficient of up to 4.0480. Higher temperatures can increase the solubility of the analyte, decrease solvent viscosity, and improve diffusion rates, thus enhancing mass transfer from the solid phase into the liquid form of extraction solvent (Mustafa and Turner, 2011). Phenolic compounds in the waste may bind more tightly to the solid sample, leading to the requirement of higher extraction temperatures compared to the *Empetrum* sample. The increased temperature may have promoted the release of these compounds from digested plant residues, microbial biomass, and organic complexes formed during transit through the digestive procedure.

Extraction time also has a certain impact on TPC recovery. According to the model, longer extraction times lead to a larger amount of phenolic compounds released. This differs from the results observed in the plant matrix above, where longer extraction times were not required. This difference in extraction time may be due to the complexity of the matrix. The plant matrix contains many complex components and compounds, which can hinder the transfer of phenolics from the matrix into the environment. Slower diffusion suggests that

phenolic compounds may be trapped in undigested plant fragments or bound to insoluble organic components, requiring longer extraction times to achieve high recovery efficiency.

Quadratic effects were observed to have a considerable effect on the optimization of the extraction process. A negative second-order coefficient for solvent concentration indicates a limit to the solvent's polarity. Thus, the solution is efficiently extracted at medium concentrations rather than at extreme limits. Meanwhile, the second-order coefficients for temperature and extraction time show the opposite. Increasing temperature and extraction time increases TPC within the experimental range. These findings suggest that the experimental limits may not fully capture the optimal extraction conditions for the matrix.

The significant differences in experimental factors between the two matrices highlight the complexity of phenolic extraction after digestive processes. During digestion, naturally occurring phenolic compounds may undergo enzymatic, microbial, or oxidative transformations, producing compounds with physicochemical properties different from the original compounds present in the consumed plant (Aura 2008). Therefore, the extraction efficiency of these compounds may be controlled by a greater number of extraction parameters. Thus, optimizing ASE conditions is particularly important for each matrix, especially those with complex effects such as faeces

Table 13 summarizes the statistical data parameters for TPC and TFC recovery using the reindeer matrix. Regarding to the model, the optimal TPC extraction parameters are predicted at extremes where the model failed to capture all the data. This is also clearly shown in Figure 9. The extraction solvent was chosen as 60% with an extraction temperature and time of 140°C and 15 minutes, respectively

Table 13: ANOVA summary table according to TPC and TFC responses of reindeer matrix coefficients of model equation, sum of squares (SS), degrees of freedom (DF), mean squares (MS), F statistic (F-value), and probability value (p-value)

	TPC					
Factor	Coefficient	SS	DF	MS	F-value	p-value
Model		285.0940	9	31.6770	44.7388	0.0003
Linear term		245.6410	3	81.8800	115.6425	0.0000
x_1	-3.4626		1		-11.6390	0.0001
x_2	4.0480		1		13.6068	0.0000
x_3	1.5262		1		5.1301	0.0037
Quadratic		25.2650	3	8.4220	11.8943	0.0103
x_1^2	-1.3507		1		-3.0844	0.0273
x_2^2	1.7501		1		3.9964	0.0104
x_3^2	1.2709		1		2.9023	0.0337
Interactions		14.1880	3	4.7290	6.6796	0.0336
x_1x_2	-1.6301		1		-3.8745	0.0117
x_1x_3	-0.7659		1		-1.8203	0.1284
x_2x_3	0.5507		1		1.3090	0.2475
Lack of fit		3.3410	3	1.1140	11.1525	0.0834
Pure error		0.2000	2	0.1000		
R^2		0.9877				

	TFC					
Factor	Coefficient	SS	DF	MS	F-value	p-value
Model		106.1490	9	11.7942	2.3272	0.0380
Linear term		65.1630	3	21.7209	4.2859	0.0755
x_1	0.3736		1		0.4694	0.6586
x_2	2.7028		1		3.3958	0.0193
x_3	0.8371		1		1.0518	0.3411
Quadratic		35.6470	3	11.8820	2.3446	0.1898
x_1^2	-1.7711		1		-1.5118	0.1910
x_2^2	1.3991		1		1.1942	0.2860
x_3^2	-2.0515		1		-1.7511	0.1403
Interactions		5.3390	3	1.7797	0.3512	0.7909
x_1x_2	-1.1430		1		-1.0154	0.3565
x_1x_3	-0.1682		1		-0.1494	0.8870
x_2x_3	0.0084		1		0.0075	0.9943
Lack of fit		24.1900	3	8.0632	14.0191	0.0673
Pure error		1.1500	2	0.5752		
R^2		0.8073				

Figure 9 illustrates the interaction model between the extraction conditions: solvent concentration, temperature, and time in the reindeer factors matrix. In contrast to the *Empetrum* matrix, the graphs tend to grow outside the investigated range for all three conditions. Combined with the above statistics, we can conclude that the optimization model worked well for this matrix; however, the survey range has not yet represented the true optimal point. In other words, the optimal point within this range lies at the edge of the survey range.

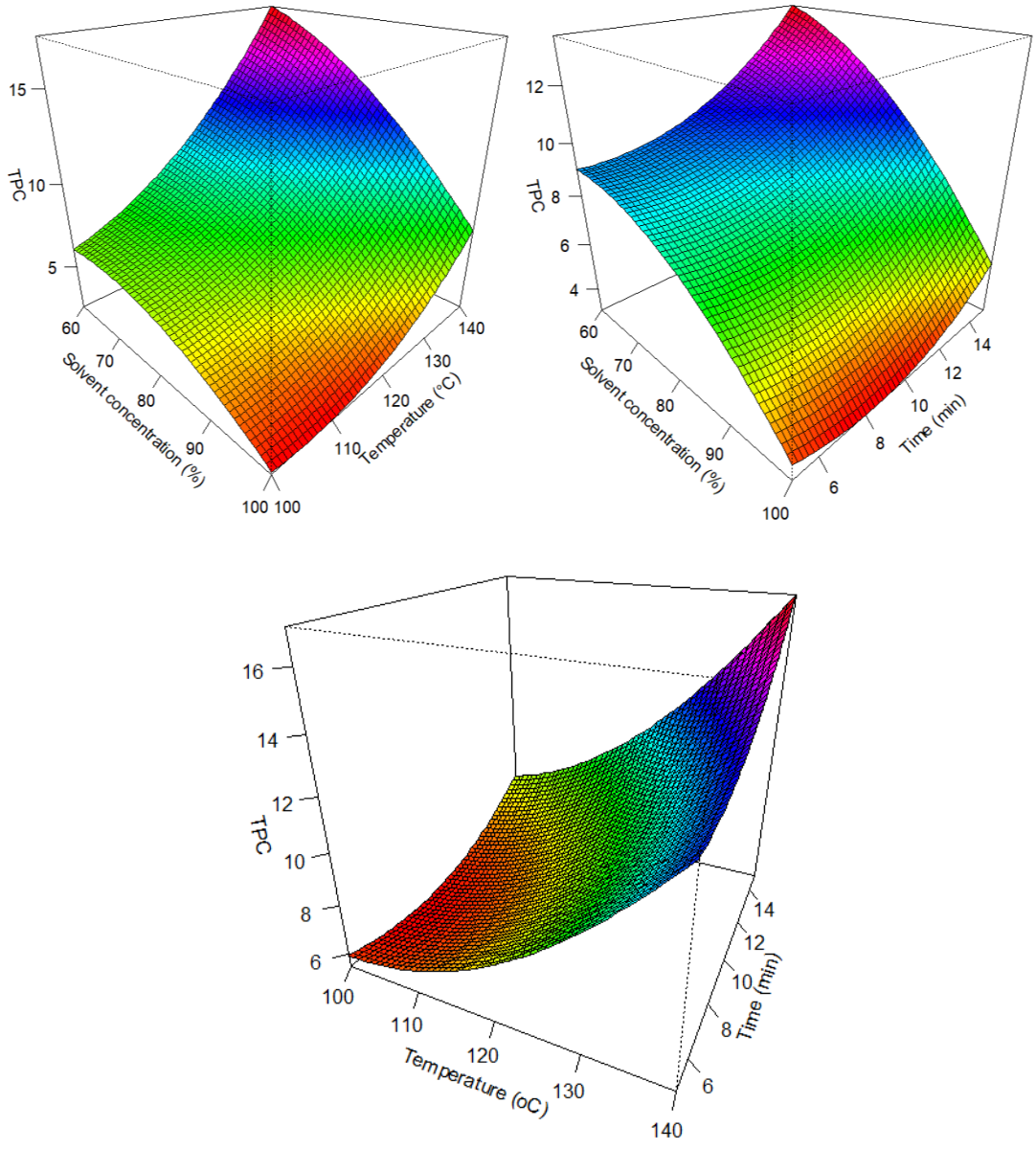


Figure 9: 3D contour of TPC response of reindeer matrix

BBD model for the extraction of TFC from reindeer faeces was statistically significant (p-value = 0.038), indicating that the selected ASE parameters contributed to the variation in flavonoid recovery. However, the coefficient of determination was lower with an R^2 of 0.8073 compared to the corresponding TPC model. Nevertheless, a p-value of 0.067 > 0.005 confirmed that this model worked well within the experimental range. Equation (9) represents the TFC recovery model.

$$TFC = 16.3607 - 1.7711x_1^2 + 1.3991x_2^2 - 2.0516x_3^2 - 0.3736x_1 + 2.7028x_2 + 0.8371x_3 - 1.1429x_1x_2 - 0.1682x_1x_3 + 0.0084x_2x_3 \quad (9)$$

Furthermore, TFC recovery efficiency was strongly affected by temperature, with a relatively high positive correlation coefficient indicating that increasing temperature promoted flavonoid extraction. Meanwhile, solvent concentration and extraction time did not have as significant an impact as temperature. Moreover, the quadratic indices of both factors showed that those parameters reached its highest values at an average level within the investigated range. This behavior differed significantly from TPC.

Overall, the results indicate that temperature is the primary factor controlling the flavonoid recovery process from reindeer faeces, while solvent composition and extraction time have relatively minor effects. Compared to TPC, the flavonoid extraction process showed less significant impact and lower model predictability. This highlights the complexity of flavonoid transformations occurring during digestion and their influence on subsequent extraction behaviour.

Based on the predictive model and 3D plot in Figure 10, the selected solvent concentration is 74.25% with an extraction temperature of 140°C and a working period of time around 12.63 minutes.

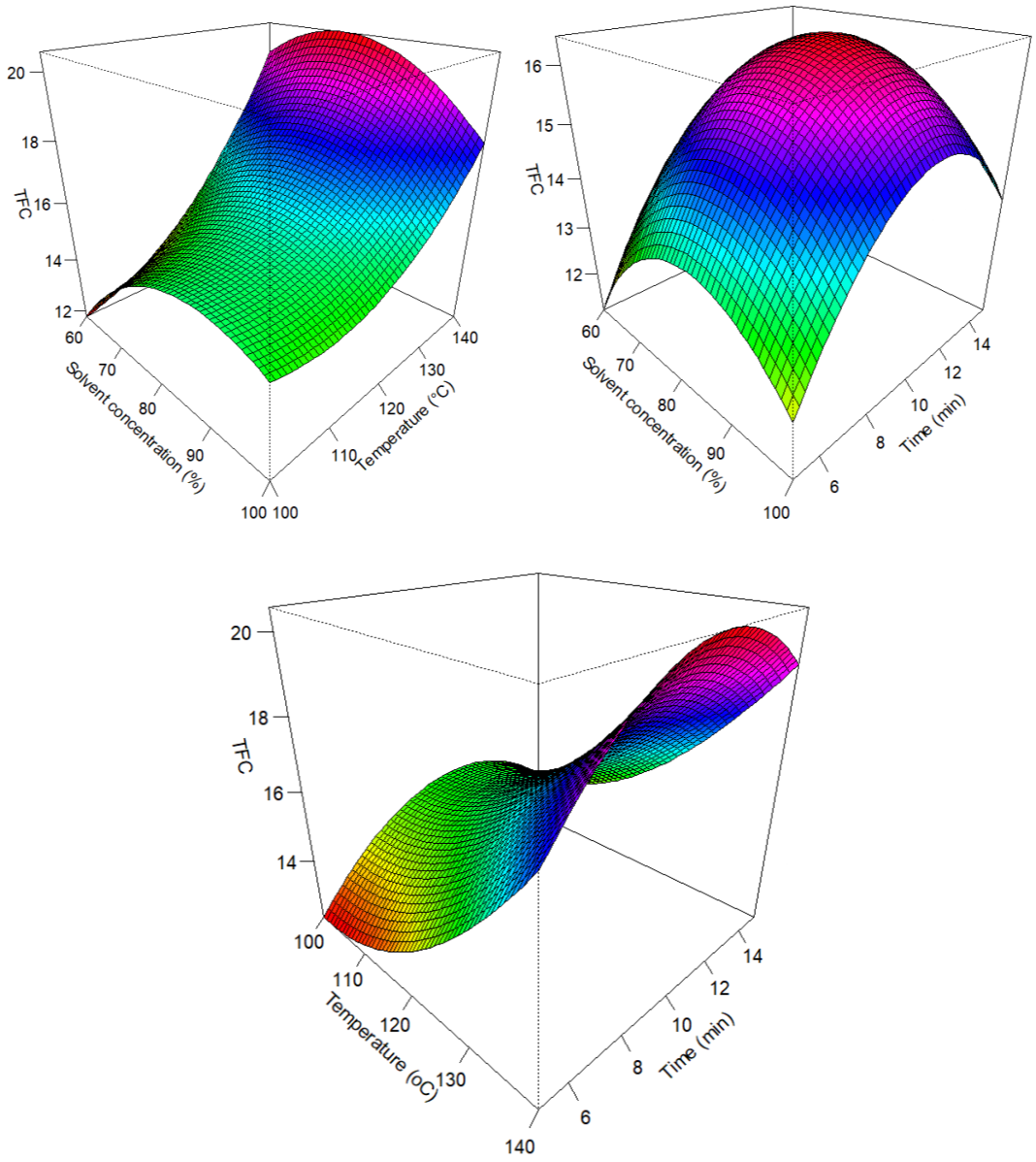


Figure 10: 3D contour of TFC response of reindeer matrix

Therefore, to achieve a balance between the TPC and TFC recovery models of the reindeer faeces matrix, the optimal extraction point is a solvent concentration of 68%, and the extraction temperature and time are 140°C and 13 minutes, respectively.

4.4 Sample extractions

4.4.1 Model validation

To verify the reliability of the RSM models, *Empetrum* and reindeer faeces matrices were extracted at the optimized extraction conditions. Both matrices yielded experimental results that closely matched the forecasted optimal values. This demonstrates the high reliability of the model.

For *Empetrum nigrum*, the predicted TPC and TFC values were 80.04 mg GAE / g DW and 57.09 mg RE / g DW, while the experimental values obtained under optimal conditions were 81.55 ± 4.07 mg GAE / g DW and 60.49 ± 4.24 mg RE / g DW. These values were all within the predicted range. Similarly, for reindeer faeces, the predicted TPC values were 21.58 mg GAE / g DW, while TFC values reached 20.09 mg RE / g DW. Corresponding experimental contents were 25.17 ± 1.03 mg GAE / g DW and 22.02 ± 1.02 mg RE / g DW.

The relatively small difference between predicted and experimental values demonstrated that the model successfully described the optimization system for the ASE extraction process. Although the experimental values differed, the difference remained within acceptable limits. These findings confirm the suitability of RSM as an optimization tool for the ASE extraction process, and the optimized conditions can be reliably applied to recover phenolic and flavonoid compounds from both *Empetrum nigrum* and reindeer faeces.

Table 14: Method validation results

Matrix	TPC predicted (mg GAE/ g DW)	TPC (mg GAE/ g DW)	TFC predicted (mg RE/ g DW)	TFC (mg RE/ g DW)
Empetrum nigrum	80.04	81.55 ± 4.07	57.09	60.49 ± 4.24
Reindeer faeces	21.58	25.17 ± 1.03	20.09	22.02 ± 1.02

4.4.2 Sample extraction results

After confirming the optimum ASE parameters, the extraction technique was employed to different material samples derived from *Empetrum nigrum* and reindeer faeces to assess differences in TPC and TFC. To obtain a comprehensive view of phenolic and flavonoid contents among each matrix, young shoots, old shoots, litter, and reindeer faces were analysed separately. Table 15 shows significant differences between the sample types, reflecting both plant growth stages.

Table 15: TPC and TFC of different matrices

Matrix	TPC (mg GAE/ g DW)	Mean (mg GAE/ g DW)	SD (n=3)	RSD (%)	TFC (mg RE/ g DW)	Mean (mg RE/ g DW)	SD (n=3)	RSD (%)
Young shoots	177.11	176.47	0.90	0.51	105.44	103.67	2.51	2.42
	175.83				101.89			
Old shoots	129.30	128.65	1.25	0.97	90.57	89.44	1.12	1.25
	129.45				89.42			
	127.21				88.33			
Litter	2357	23.10	1.09	4.72	21.90	21.90	0.48	2.20
	23,88				21.42			
	21,.86				22.39			
Reindeer faeces	25.33	25.17	1.03	4.11	20.94	22.02	1.02	4.65
	24.06				22.97			
	26.11				22.16			

In all matrix samples, young shoots exhibited the highest concentrations of phenolic and flavonoid compounds. TPC was 176.47 mg GAE/g DW, while TFC value reached 103.67 mg RE/g DW. This is entirely logical as young shoots are vigorously developing, and phenolic compounds are a major contribution in plant defence and growth. Therefore, as the plant develops, phenolic compounds can be continuously regenerated and produced to sustain the plant's life.

Both TPC and TFC responses witnessed a decrease in old shoots matrix, with values dropping to 128.65 mg GAE/g DW and 89.44 mg RE/g DW. This difference might be explained by physiological changes during tissue maturation, or the redistribution of metabolic resources and reduced biosynthesis of protective compounds. This decrease has

been compared in several studies, in which young tissues generally contain higher concentrations of bioactive compounds than older tissues (Wallsted et al 1997).

Furthermore, the above results are also considered reasonable when put in comparison with the outcomes of Väisänen et al (2013). According to their study, the total amount of soluble phenolic compounds recorded in *Empetrum* leaves was 86.7 ± 2.80 mg/g DW, slightly lower than the thesis' experimental numbers. However, Väisänen's report only focused on a few main target groups for quantification using the HPLC-DAD method. Flavones, flavanols, benzoic acid, chlorogenic acid, protocatechuic acid, batatasin-III, stilbenes, and condensed tannins were the groups of compounds of interest (Väisänen et al 2013). Meanwhile, this thesis used the Folin-Ciocalteu reagent method to determine TPC in all the groups of compounds present in the extract. This reagent works based on electron transfer and can be affected by other reducing substances, which suggests that other phenolic compounds, sugars, amines, or amino acids may have been involved in the measured signal. Therefore, the TPC recorded in the thesis has a higher correlation index but is still within an acceptable and explainable range.

The most significant reductions were observed in litter samples. TPC decreased to 23.10 mg GAE/g DW, with TFC reaching 21.90 mg GAE/g DW. This could be due to decomposition processes occurring after the plants age, contamination by the harvesting environment, or the leaching of soluble compounds caused by environmental exposure. Unlike reindeer faeces, which may contain residues of various other plant species and microbial biomass, the litter matrix tends to contain a higher proportion of *Empetrum*-derived compounds since the litter is composed directly of *Empetrum nigrum* tissue. However, it will still be complex due to contamination by the soil and the surrounding environment. This means that TPC and TFC may be underestimated or diluted by various factors.

Interestingly, the TPC and TFC values obtained from reindeer were comparable to those observed in the litter matrix. This result suggests that phenolics and flavonoids from plants that consumed by reindeer may persist after passing through the digestive tract and remain detectable after excretion. However, digestion partially degraded the bioactive compounds, which indicates the decrease in TPC and TFC in this matrix. Furthermore, the phenolic and flavonoid compounds may originate from various sources other than *Empetrum nigrum*. The results of detecting batatasin-III in reindeer faeces samples shown in section 4.5.2 will be discussed further in this regard.

Statistically, low relative standard deviation (RSD) values were observed across all matrices. The recorded values ranged from 0.51% to 4.72% for TPC and from 1.25% to 4.65% for TFC, indicating good repeatability of the optimized ASE method.

Overall, the results showed a notable decrease in phenolic and flavonoid content from young shoots, mature shoots, litter matter, and reindeer faeces. However, the persistence of TPC and TFC in reindeer faeces highlighted the ecological importance of this matrix to the environment, as phenolic metabolites persisted during digestion and were transported into the soil environment.

4.5 GC-MS

4.5.1 *Empetrum nigrum* profiling

GC-MS analysis of ASE extracts obtained from young shoots of *Empetrum nigrum* revealed a diverse chemical profile including organic acids, sugars, amino acids, alcohols, phenolic acids, flavonoids, stilbenoids, bibenzyl derivatives, and terpenoids. Table 16 summarizes the organic compounds detected in the young shoot matrix of *Empetrum nigrum*. Figure 11 is a GC-MS plot of these compounds, including distinct peaks.

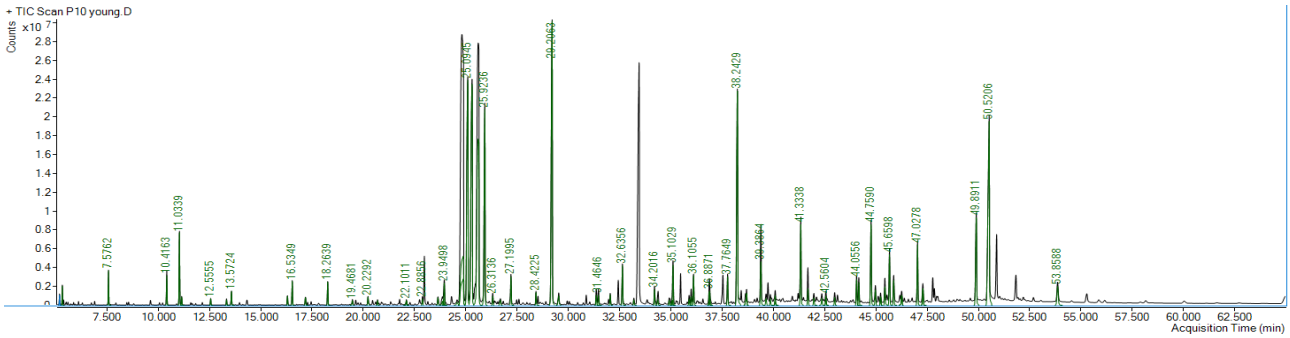


Figure 11: Metabolites profiling of *Empetrum nigrum*

Regarding to the qualitative perspective, a significant portion of the identified compounds are organic acids, including methylphosphate, glycerate, malate, citrate, quinate, erythronate, threonate, palmitate, linoelaidic, and stearate. The presence of malic acid, citric acid, and quininic acid has been noted in *Empetrum nigrum*, particularly in the fruit and

black crowberry juice (Laaksonen et al. 2011). Citric acid plays a role in the characteristic sour and bitter taste depending on its concentration (Laaksonen et al. 2011). This can be considered a defence mechanism of the plant against herbivores.

Furthermore, an indispensable component of the black crowberry matrix is sugars. The sugar compounds found include fructose, mannose, sucrose, maltose, and several monosaccharide derivatives. These products are the plant's energy reserves and precursors for the biosynthesis of secondary metabolites. Furthermore, carbohydrates contribute the carbon framework for the biosynthesis of phenolic and flavonoid compounds through interconnected metabolic pathways (Taiz et al., 2015).

Among the secondary metabolites, phenolic acid compounds are one of the most important and extensively studied groups of *Empetrum nigrum*. Benzoic acid, protocatechuic acid, and chlorogenic acid have been identified as the dominant phenolic acids. These phenolic compounds account for the antioxidant and antimicrobial activities of this plant. All three compounds have been noted in studies on *Empetrum nigrum*, which demonstrates that these phenolic compounds are fundamental and commonly found in this dwarf shrub (Ekaterina et al 2021). In addition, several other typical compounds in crowberries have also been studied, such as glycerol, myo-inositol, catechin, beta-sitosterol, and ursolic acid (Muravnik et al 2012).

Notably, the discovery of batatasin-III, a bibenzyl compound, has been repeatedly linked to the ecological success of *Empetrum nigrum*. Batatasin-III has attracted scientific attention due to its growth-inhibiting properties and ability to prevent seed germination. Previous studies have also addressed this issue, with batatasin-III contributing to the superior growth of *Empetrum nigrum* in adjacent ecosystems, reducing the formation of competing vegetation (Nilsson et al 1993; González et al 2014). The detection of batatasin-III in young shoots is predictable given its high concentration (Wallsted et al. 1997). Section 4.5.3 will present more details on the quantification of this compound in the shoot matrix.

Overall, GC-MS analysis results showed that young shoots of *Empetrum nigrum* contained a broad spectrum of primary and secondary metabolites. Besides unknown peaks, the identified compounds supported previous reports describing *Empetrum nigrum* as a rich source of bioactive compounds and provided the chemical basis for its antioxidant,

antimicrobial, and growth-inhibiting properties. Furthermore, the identification of batatasin-III in the extract demonstrated its importance to the ecosystem.

Table 16: metabolites detected in *Empetrum nigrum* young shoots

Group	Compounds	RT (min)	Identification
Organic acids	Methylphosphate	11.0051	a,b
	Glycerate	12.5555	a,b
	Erythronate	13.5730	a,b
	Malate	16.5364	a,b
	Threonate	18.2415	a,b
	Citric acid	23.9238	a,b
	Quinate	24.7644	a,b
	Palmitate	28.4029	a,b
	Linoelaidic acid	31.3573	a,b
	Stearate	31.9441	a,b
Sugars	Fructose	25.0185	a,b
	Mannose	25.5265	a,b
	Allose	25.8588	a,b
	Talose	27.1794	a,b
	D-ribose	35.0771	a,b
	Succrose	38.1979	a,b
	Maltose	38.6884	a,b
	Ethyl .alpha.-D-glucopyranoside	39.3864	a,b
Phenolic acids	Benzoate	10.3977	a,b
	Protocatechoic acid	23.9138	a,b
	Chlorogenic acid	44.7328	a,b
Alcohols	Glycerol	11.1427	a,b
	Myo-Inositol	29.1499	a,b
	n-Tetracosanol	39.8489	a,b
	n-Hexacosanol	42.5590	a,b
Bibenzyls	Batatasin-III	33.4480	a,b
Stilbenoids	Pterostilbene	36.0748	a,b
Flavones	Catechine	41.3087	a,b
Terpenoids	beta.-Sitosterol	47.2924	a,b
	Ursolic acid	50.4267	a,b
Amino acid	Serine	13.3309	a,b
	Pyroglutamic acid	17.1816	a,b
	Aminobutanoic acid	17.4570	a,b
	Asparagine	20.6985	a,b
	L-Tyrosine	26.3197	a,b

a – NIST library, b - Retention index of the reference compounds

Table 17 summarizes the additional compounds found in the *Empetrum* matrix of old shoots. Almost all the compounds in Table 12 were found in the old shoot matrix; however, several other compounds were also identified, adding to the diversity of this plant species. Lactate, hydroxyacetate, xylose, arabinitol, xylitol, sorbitol, 1-octadecanol, 1-octacosanol, and α-amyrin were determined.

The presence of these additional metabolites has supplemented the chemical composition data for *Empetrum*. Furthermore, these compounds have also been reported to be found in *Empetrum* nigrum via GC-MS analysis (Muravnik et al 2012). The detection of some other compounds in old shoots but not in young shoots can be explained by two possibilities. Firstly, some compounds are produced as the leaves age and play a role in the plant growth. However, this requires further study of leaf activity at each stage. Secondly, these compounds are always present in the shoot matrix, but their concentrations are too low to be detected in young shoots. As the plant develops, the concentrations of these compounds increase sufficiently to be detectable after methoximation and derivatization processes. This is understandable, as the metabolic profile of plants is very dynamic and can change depending on the plant's developmental stage.

Table 17: Additional metabolites detected in old shoots

Group	Compounds	RT (min)	Identification
Organic acids	Lactate	5.9392	a,b
	Hydroxyacetate	6.2897	a,b
Sugars	Xylose	39.7600	a,b
Alcohol	Arabinitol	21.8317	a,b
	Xylitol	21.8522	a,b
	Sorbitol	26.1204	a,b
	1-Octadecanol	30.4531	a,b
	1-Octacosanol	45.1005	a,b
Terpenoids	Alpha.-Amyrin	48.0324	a,b

a – NIST library, b - Retention index of the reference compounds

Regarding the litter matrix, the chemical composition within the matrix appears more complex, containing numerous unknown peaks and chemical fragments. However, the analysis results are encouraging, showing that the main compounds of *Empetrum nigrum*

were all found, including benzoate, lactic acid, glycolic acid, malic acid, xylitol, citric acid, quininic acid, palmitic acid, stearic acid, beta-sitosterol, alpha-amyrin, ursolic acid, and sugar compounds such as glucose, mannose, and sucrose. This demonstrates the reproducibility and consistency of the method in detecting common compounds across matrices. Furthermore, the results also demonstrate the stability of phenolic compounds across different growth stages.

4.5.2 Reindeer faeces profiling

Similar to the litter matrix, the reindeer extract matrix has a high complexity. This matrix may contain fats, lipids, digestive aids, residual plant fragments, and other components. Therefore, to focus on the research topic of *Empetrum nigrum* and related compounds, only the corresponding bioactive compounds are mentioned and listed as in Table 18.

The compounds listed below are all validated and compared with compounds detected in *Empetrum nigrum* according to the study by Muravnik et al (2012). Typical compounds were found, including organic acids, sugars, phenolic acids, alcohols, bibenzyls, terpenoids, and amino acids. The detection of these compounds suggests that certain phenolic constituents derived from consumed plant material remained detectable after passage through the digestive tract. However, the retention times of the specific compound among *Empetrum nigrum* and reindeer faeces matrices are different. This result can be explained by the complexity of the matrix; bioactive compounds are more likely to bind tightly to substances in the reindeer matrix. This could hinder derivatization and analysis with GC-MS.

And once again, the importance of detecting batatasin-III in the faecal extract is emphasized. This compound was previously detected in above-ground parts of *Empetrum nigrum* and is the compound that represented its characteristic allelopathic properties. Its presence in reindeer faeces suggests that at least part of the compound, or compounds sufficiently similar to be identified as batatasin-III by GC-MS, persists after digestion. This finding is predictable given the hypothesis that phenolic compounds are highly persistent and capable of transferring from plant to herbivorous waste through digestion.

In summary, although the phenolic composition in reindeer faeces extract is less diverse than that of the plant matrix, the detection of batatasin-III demonstrates that characteristic

metabolites of *Empetrum nigrum* can still be detected after ingestion. While ingestion may partially degrade the bioactive compounds, their persistence has been demonstrated. These results highlight the possibility that reindeer may consumed *Empetrum nigrum* leaves by accident and the method used in this thesis is effective and suitable to study herbivore-plant interactions. The procedure of ASE-GC-MS has successfully extracted and detected the batatasin-III content in investigated sample. This approach would contribute to understanding under which conditions reindeer may eat *Empetrum* plant and deepen our understanding of grazing patterns in northern region. environment. Section 4.5.3 will provide more details on its concentrations after excretion.

Table 18: Metabolites detected in reindeer faeces matrix

Group	Compounds	RT (min)	Identification
Organic acids	Lactate	5.9545	a,b
	Glycolic acid	6.3041	a,b
	Methylphosphate	7.9270	a,b
	Glycerate	12.5369	a,b
	Pyruvate	13.3266	a,b
	Malate	16.5116	a,b
	Quinate	24.7119	a,b
	Gallic acid	26.6738	a,b
	Palminate	28.3981	a,b
	Stearate	31.9464	a,b
Sugars	Fructose	20.5876	a,b
	Glucose	25.4895	a,b
Phenolic acids	Benzoate	10.3920	a,b
	Protocatechoic acid	23.8893	a,b
Alcohols	Glycerol	11.1230	a,b
	Arabinitol	21.8374	a,b
	Sorbitol	26.1234	a,b
	n-Hexadecanol	26.7845	a,b
	Myo-Inositol	29.1382	a,b
	n-Tetracosanol	42.5423	a,b
	n-Hexacosanol	42.5423	a,b
	1-Octacosanol	45.0832	a,b
Bibenzyls	Batatasin-III	33.4480	a,b
Terpenoids	Phytol	30.7142	a,b
	beta.-Sitosterol	47.2699	a,b
	Ursolic acid	50.4165	a,b
Amino acid	L-5-Oxoproline	17.1581	a,b

a – NIST library, b - Retention index of the reference compounds

4.5.3 Batatasin-III concentrations

To quantify the batatasin-III content in the investigated matrices, a calibration curve was constructed. Seven different batatasin-III concentrations were used with 1,6-dihydroxynaphthalene as the internal standard. The procedure and construction are listed in the Materials and Methods section. The calibration curve yielded the equation y = 1.2053x + 0.4816. The R^2 coefficient of 0.995 indicates a high degree of linear reliability of the equation, statistically speaking. Figure 12 depicts the constructed standard curve of batatasin-III.

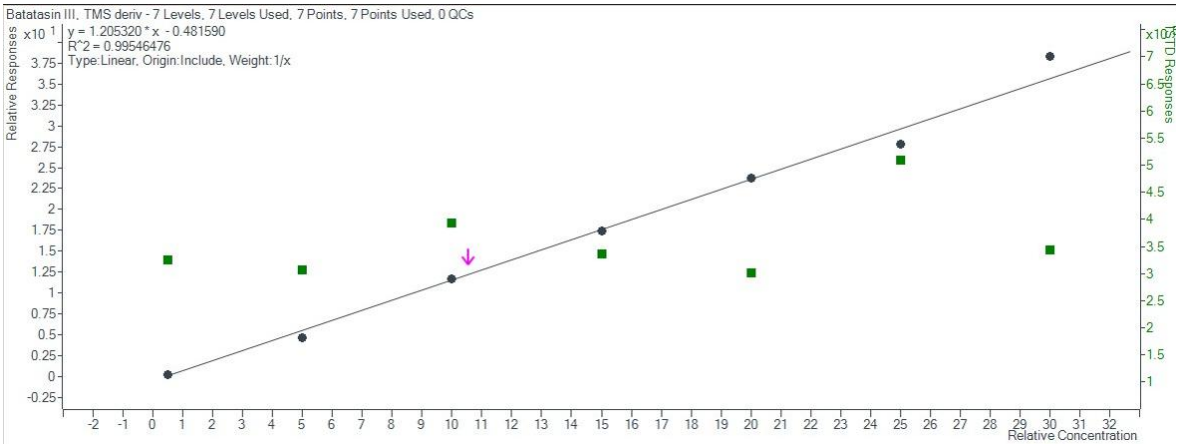


Figure 12: Calibration curve of batatasin-III

The *Empetrum nigrum* extracts were first qualitatively analysed to observe the intensity of the peaks. The black line represents the peaks of the young shoots matrix. The red and green patterns are the chromatographs of the old shoots and litter matrices, respectively. In this section, batatasin-III will be observed and discussed. As the retention time (RT) of batatasin-III was specified above, the peaks at 33.448 minutes were determined. As shown in Figure 13, the intensity of the chromatographs from young shoots and old shoots is very sharp and almost coincident, suggesting that the batatasin-III content in the young shoots and old shoots matrices is nearly equal or negligibly different. Meanwhile, the batatasin-III content in the litter matrix is predicted to be much lower than the other two matrices, as the green line has relatively wide and small peaks.

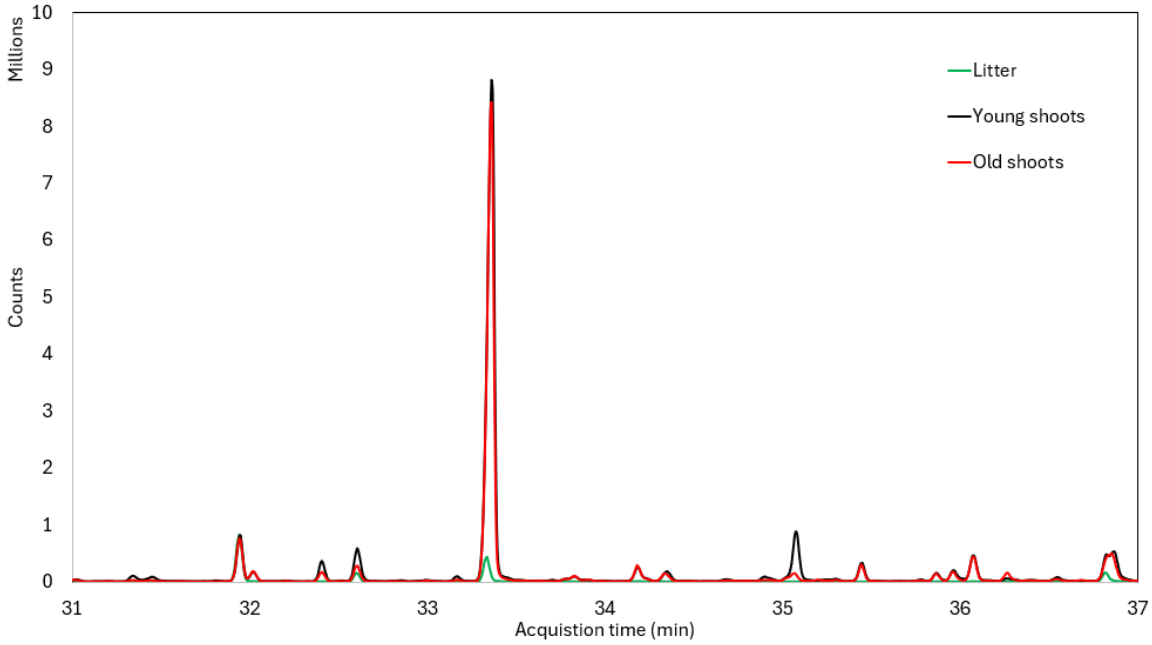


Figure 13: Chromatograph plot between young shoots (black), old shoots (red), and litter (green)

Subsequently, the extracted samples were quantitatively analysed for batatasin-II using a GC-MS instrument. Table 19 provides the data on the batatasin-III content in each different matrix. Batatasin-III concentrations showed notable variation among the study samples. The highest concentrations were witnessed in young shoots (10.67 ± 0.35 mg/g DW) and old shoots (10.69 ± 0.43 mg/g DW), while significantly lower concentrations were detected in litter (0.76 ± 0.17 mg/g DW) and reindeer faeces (0.40 ± 0.01 mg/g DW). However, the *Empetrum* plant sample, including young shoots, old shoots, and litter, used both leaves and stems in the extraction process. Therefore, if only the batatasin-III concentration in the leaves is considered, those values may be higher.

Besides, the phenomenon of equivalent batatasin-III content in young and old shoots has also been demonstrated in several related studies (Gallet et al 1999, Wallsted et al 1997), although these studies focused on *Empetrum hermaphroditum*, a species closely related to *Empetrum nigrum*. Observations showed no difference in batatasin-III content in one-year and two-year leaves, and this content only decreased significantly when leaves were analysed at three to four years old. Therefore, equivalent concentrations of batatasin-III were observed in both young and old shoots, indicating that the age of the investigated leaves

recorded could be in the first year for young shoots and second or third years for old shoots at the time of harvest, and batatasin-III was consistently present throughout the plant's growth cycles. Although TPC and TFC decreased between young and mature shoots, batatasin-III concentrations remained relatively stable. This suggests that the compound is a persistent component in plant metabolism and plays an essential part in the ecosystem.

Furthermore, an interesting observation was found in the study by Väisänen et al (2013) that while the total soluble phenolic content reached approximately 86.7 ± 2.8 mg/g DW, the batatasin-III content in the leaves was only about 5.2 ± 0.5 mg/g DW. This means that among the target TPCs, batatasin-III accounted for about 6%. This figure demonstrates the reliability and feasibility of the ASE extraction and analysis methods in this thesis. The TPC of young shoots recorded above was 176.47 ± 0.90 mg GAE/g DW, while batatasin-III content accounted for about 6% with a value of 10.67 ± 0.35 mg/g DW. In contrast, for old shoots, the relative contribution of batatasin-III was slightly higher, accounting for approximately 10% of the TPC. Although caution is needed when making direct comparisons due to differences in analytical methods and sample characteristics, the observed equivalence suggests that batatasin-III accounts for a relatively stable share of the total phenolic content in the *Empetrum* plant tissue.

Meanwhile, the litter sample had a relatively low batatasin-III content, only about 10% compared to the *Empetrum nigrum* shoot sample. Batatasin-III content was recorded in the litter as 0.76 ± 0.17 mg/g DW, which is quite similar to the previously observed result of 1.2 ± 0.1 mg/g DW in the study by Wallsted et al (2000). The decrease between young shoots and litter may be caused by the microbial decomposition of the leaves, the long harvesting time, and environmental leaching (Gallet et al 1999). However, a small amount of batatasin-III could still be found, indicating that this compound is partially decomposed, while other bioactive compounds have been severely degraded.

Batatasin-III was found at the lowest concentrations in reindeer faeces samples. This reflects the influence of digestion on organic compounds. However, quantitative analysis revealed that batatasin-III levels were lower than the linear range. Therefore, SIM mode was run when reconstructing the calibration curve and re-analysing reindeer faeces samples to quantify samples containing extremely low levels of batatasin-III. Figure 14 shows the new calibration curve when running in SIM mode.

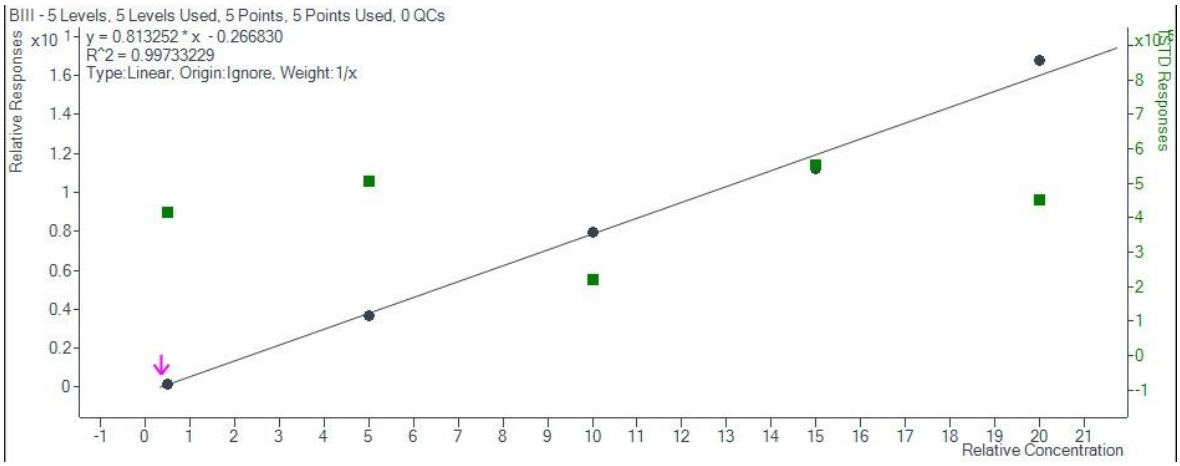


Figure 14: Calibration curve of batatasin-III under SIM mode

The calibration curve with SIM was investigated with batatasin-III concentrations ranging from 5 ng/uL to 200 ng/uL. The R^2 value was quite high, reaching 0.997, ensuring the reliability of the model. LOD and LOQ were calculated to be 23.58 ng/uL and 71.45 ng/uL, respectively. Meanwhile, the batatasin-III value in the reindeer faeces sample provided by GC-MS was only about 3.3 ng/uL, lower than the detection threshold. Therefore, although we can demonstrate that batatasin-III still exists in the reindeer faeces extract, its content could not be quantified. To my knowledge, no previous studies have researched the occurrence of batatasin-III in reindeer faeces or investigated its fate during digestion. The detection of batatasin-III in the faecal matrix indicates that reindeer may consume some amount of *Empetrum nigrum* and that batatasin-III may not be completely metabolized during digestion.

Thus, the presence of batatasin-III in both litter and reindeer faeces matrices demonstrates its long-term persistence in the environment. The translocation of batatasin-III from *Empetrum nigrum* into the soil environment via litter proves its importance in the development of vegetation ecosystems. Batatasin-III was detected in reindeer faeces samples, which provided new perspectives into the transfer of plant metabolites through herbivore digestive systems. Those findings have made meaningful contributions to reindeer ecology and pasture ecology for further comprehensive research.

Table 19: Batatasin-III concentration in different matrices

Matrix	Batatasin-III concentration (mg / g DW)
Young shoots	10.67 ± 0.35
Old shoots	10.69 ± 0.43
Litter	0.76 ± 0.17
Reindeer faeces	Detectable

4.6 Limitations and recommendations

The results of profiling and quantification of batatasin-III content have shown potential for research into the bio pathogenesis of this compound, especially as it has been shown to have certain effects on vegetation and the environment. However, several issues need to be considered and further researched.

First, while conducting this study, two correlation results between the matrices were recorded that can be compared: TPC and TFC and the measured batatasin-III content. As shown above, although the TPC and TFC of the old shoots matrix showed a significant decrease compared to the young shoots matrix, there was no difference between the two matrices in batatasin-III content. This is quite interesting, showing that high TPC or TFC concentrations do not necessarily mean high batatasin-III content. Therefore, if we want to study the optimization of the batatasin-III recovery process in *Empetrum nigrum*, TPC and TFC are not yet reasonable responses. Batatasin-III concentration is suggested to be used directly in the extraction optimization process to recover batatasin-III and for further research.

Second, due to the matrix's high complexity and insufficient time, the matrix effect was not taken into account. According to the study by Ryde et al (2025), the matrix has a certain influence in the quantification of batatasin-III. Ryde suggested that the matrix leads to underestimation in the results by 13-18%. Thus, batatasin-III in the young shoots, old shoots, litter, and reindeer faces matrices may have higher concentrations than those reported in this study. In particular, the higher the matrix complexity, the greater the likelihood of

underestimating the target compounds' content. Therefore, this should be considered when conducting further studies.

In summary, the results in this thesis describe the potential of applying the accelerated solvent extraction method to extract *Empetrum nigrum* and reindeer faeces, given that the metabolite profile has been captured quite thoroughly and in detail. However, for further in-depth study of batatasin-III, some modifications are suggested.

5 Conclusion

This thesis successfully studied and optimized accelerated solvent extraction (ASE) conditions for the recovery of phenolic and flavonoid compounds from *Empetrum nigrum* and reindeer faeces. Response surface methodology was applied and proved effective in optimizing the extraction process, as it was able to assess the variability and interactions between the investigated factors. The models yielded good results, demonstrating reliable predictive capabilities, and the experimental results were validated within acceptable limits. This demonstrates the strong reliability and reproducibility of the optimization method. Thus, ASE has proven to be a green alternative extraction method with lower solvent usage and shorter extraction times compared to existing methods, such as maceration and Soxhlet.

Solvent concentration and extraction temperature have been shown to have a certain dynamic impact on optimizing ASE methods. Solvent concentration plays a dominant part in the release of organic compounds with different polarities within the sample matrix. Meanwhile, temperature promotes the transport and release of these compounds from the plant into the extract. ASE, with its advantage of precise control of operational temperature and pressure, helps shorten extraction time and minimize its impact on the final results.

Young shoots of *Empetrum nigrum* had the highest TPC and TFC values reached at 176.47 ± 0.9 mg GAE / g DW and 103.67 ± 2.51 mg RE / g DW, respectively, followed by old shoots, while significantly lower concentrations were observed in litter samples and reindeer faeces. A slight decrease was witnessed in old shoots compared to young shoots with TPC at 128.65 ± 0.97 mg GAE / g DW and TFC at 89.44 ± 1.12 mg RE / g DW. *Empetrum´*s litter and reindeer faeces had relatively similar total phenolic and flavonoid content around 23.10 ± 1.09 mg GAE / g DW and 21.90 ± 0.48 mg RE / g DW, respectively. These findings suggest a gradual depletion of extractable phenolic compounds from living plant tissue into decomposed and digested materials. However, measurable amounts of phenolics and flavonoids were still detectable in both the litter and faeces samples, indicating that some of these compounds persisted after decomposition and digestion.

GC-MS analysis revealed complete data on primary and secondary metabolites in *Empetrum nigrum*. Several characteristic compounds were identified, with batatasin-III being of particular interest. The synthesis and enumeration of these compounds demonstrate that

Empetrum nigrum is a plant species with a rich source of bioactive compounds with potential applications in ecology and pharmaceuticals.

One of the most important findings of this study is the detection and quantification of batatasin-III in all *Empetrum* plant samples surveyed and detection of batatasin-III in faeces matrix. High concentrations were observed in young shoots and old shoots with the value of around 10 mg / g DW. This is understandable, as it is a characteristic compound found in fresh shoot samples. Meanwhile, low concentrations were found in litter at 0.76 mg / g DW and reindeer faeces. However, the presence of batatasin-III in faecal samples suggests that reindeer may have consumed *Empetrum nigrum*, albeit unintentionally, since crowberries are dominant in the vegetation of tundra pastures, and this compound is not completely degraded during digestion and after excretion. These results support contributions to future research on reindeer and pasture ecology. To our knowledge, this is the first study investigating the occurrence and persistence of batatasin-III from above-ground parts of *Empetrum nigrum* in reindeer faeces samples.

Overall, the study has met and addressed all the criteria set at the beginning of the thesis. This contributes to bridging the existing knowledge gap on systematic ASE optimization for *Empetrum nigrum* and reindeer faeces, while providing new insights into the occurrence and persistence of phenolic compounds, particularly batatasin-III.

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Appendix: Standard curves for total phenolic content (TPC) and total flavonoid content (TFC) determination

Gallic acid calibration curve

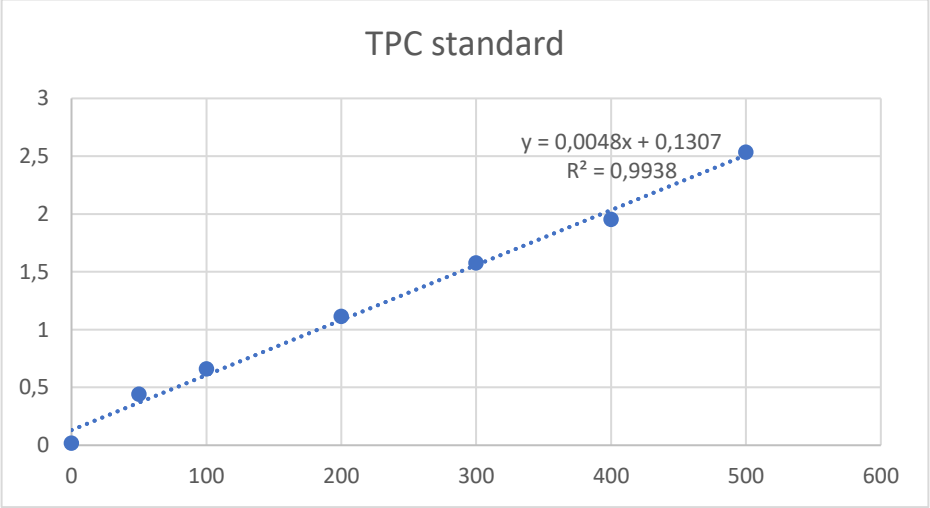

Figure A1: Gallic acid calibration curve used for TPC determination.

Rutin calibration curve

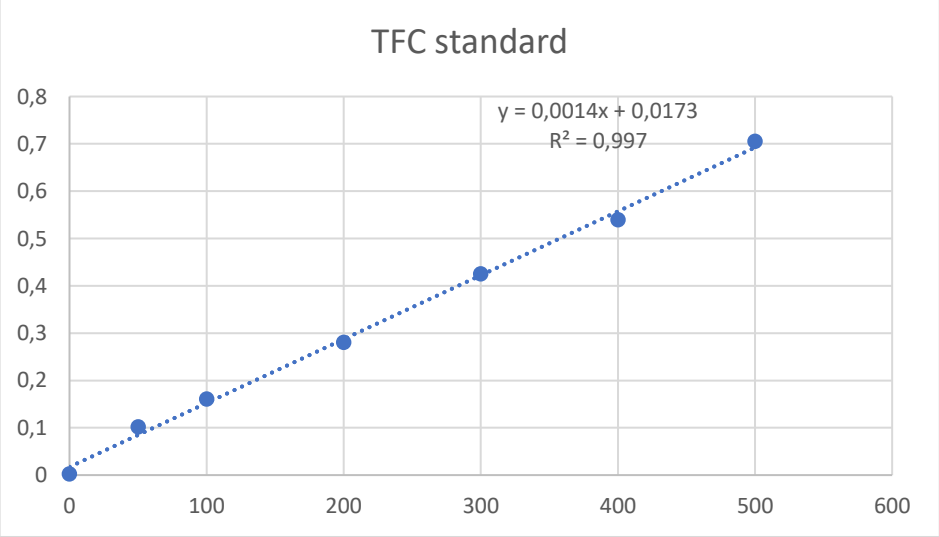

Figure A2: Rutin calibration curve used for TFC determination.