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Assessing The Performance of NIR Spectroscopy in the Quantification of Polyphenols in Grape Seed and Other Plant Extracts

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1. INTRODUCTION AND OBJECTIVES

This section very briefly summarizes context and challenges surrounding the valorization and authentication of GSE and defines the specific goals of the thesis.

Agri-food waste utilization is an important aspect of sustainability. Grape seed as a byproduct of wine and grape industry is valorized into grape seed extract, which is one of the most broadly used botanical extracts, popularly purchased by consumers in the form of dietary supplements.

GSE is found to be prone to adulteration with other proanthocyanidin rich substances, like pine bark extract, peanut skin extract and green tea extract. This tampering can go undetected throughout the food chain, due to the application of conventional analytical methods without in-depth profiling, the use of which is time consuming, usually destructive to the sample and costly. The high chemical similarity of adulterants suggests that non-targeted, fingerprinting methods evaluating broad physicochemical properties are most efficient. From this category, near-infrared spectroscopy is a possible non-destructive, green, fast and cost-effective vibrational technique with existing portable designs enabling on-site real time assessments of quality.

Based on this, the primary objective of this thesis is assessing the performance of NIR spectroscopy in the quantification of polyphenols in grape seed and other plant extracts. For this the following subtasks were completed:

- Preparing pure GSE, PBE, PSE and GTE samples as well as adulterated GSE mixtures with ten additive levels in line with the industrial practice of adulteration and conducting measurements on the resulting samples with a benchtop NIR device.
- Characterizing GSE and its adulterants with antioxidant capacity determination, total polyphenol content determination and HPLC analysis.
- Quantifying extract concentrations and chemical parameters with predictive models built on spectral data using chemometric methods.

2. LITERATURE REVIEW

After defining the main objectives, this section provides essential background on agri-food waste and its valorization, with particular emphasis on the wine industry's byproducts, above all grape seed extract (GSE), its relevance and the challenges surrounding its authentication. It also explores the issue of adulteration in the food industry, outlining the main analytical techniques used to identify it, with a specific focus on chromatographic and spectroscopic methods.

2.1. Importance of agri-food waste utilization in sustainability

On a global scale, food waste is among the most prevalent forms of biowaste produced today. Statistical data indicates food and beverage production, distribution, and retail contribute to roughly 20% of the total 14 MMT of waste generated annually (Sarker et al., 2024). As a concept food waste can be broadly characterized as „wholesome edible material intended for human consumption, arising at any point in the food supply chain that is instead discarded, lost degraded or consumed by pests" according to FAO (1981), while this characterization can be expanded by „ also edible material that is intentionally fed to animals or is a byproduct of food processing diverted away from the human food" stated by Stuart (2009) and „including overnutrition, being the gap between the energy value of consumed food per capita and the energy value of food needed per capita" in accordance with Smil (2004) (Parfitt et al., 2010).
In respect to agri-food waste management Papaioannou et al. (2022) found that nowadays it is estimated that more than 1.3–1.6 billion tons of food are thrown away along the entire food supply chain worldwide, which is roughly equal to one-third of the global food production for human consumption and more than one quarter of the global agricultural production. Interestingly, among various food processing sectors, the fruit and vegetable industry generate the largest proportion of waste, contributing approximately 25–30% of the total. This waste includes peels, rinds, seeds, cores, rags, stones, pods, vines, skins, and pomaces (Sarker et al., 2024). Meanwhile in the European Union it is estimated that the beverage industries account for around 26% of the total food waste, making them the largest contributing asset, proceeded by the dairy industry, fruit and vegetable industry and cereal industry with 21%, 14.8% and 12.9%. Consequently, to eliminate or significantly reduce food waste has become a major focus for society and governments globally, given its significant economic, social, health, and environmental impacts (Papaioannou et al.,2022).

The sustainable development of food companies by making efforts to optimize food processing technology and therefore minimize waste is in focus nowadays. A crucial part of this is the use of inevitable byproducts also known as food valorization that confers added value to these waste materials (Salazar et al., 2018). The proper management of food waste streams by valorization can be a keyway in achieving a win-win circular economy. The mentioned streams derived from the food industry are considered sources of diverse nutrients and bioactive compounds (Sarker et al.,2024). Furthermore, carbon retains in food industry wastes its organic form rather than being released as CO_2. The discussed waste streams typically contain a diverse range of organic compounds, which can be categorized into several predominant groups, which include: carbohydrates (such as pectins, oligosaccharides, starch, cellulose, dietary fibers and monosaccharides); biophenols (like lignin, phenolic acids, flavonoids, tannins and ellagitannins); proteins; lipids; essential oils (comprising terpenoids, hydrocarbons, aliphatic alcohols and ethers, lactones and polyacetylenes); and pigments (including anthocyanins, carotenoids, betalains and chlorophyll). These agri-food byproducts are proven to be beneficial raw materials in the production of a diverse array of intermediates with potential uses as food ingredients, cosmetics, materials, biofuels or biopolymers (Papaioannou et al.,2022; Ben-Othman et al.,2020).

The extraction of potential high-value bioactive compounds can be distinguished into conventional and green methods with diverse advantages and limitations shown on **Figure 1**. Regardless of the method used, all extractive techniques share common aims:

1. to extract the desired target compounds, minimizing their degradation, and without altering their properties;
2. to obtain the extract in the most stable and pure form;
3. to increase the extraction yield of the desired compounds;
4. and to reduce costs and process time (Sorrenti et al.,2023).

In the sustainable bioconversion of fruit and vegetable waste conventional extraction techniques, such as intensive grinding, maceration, soaking, Soxhlet and thermal processing are increasingly being phased out due to their high energy consumption and cost inefficiency. As a result, several modern extraction methods have been developed over time, offering green and sustainable alternatives for converting agricultural waste into valuable resources. These advanced techniques include microwave-assisted and ultrasound-assisted extraction, supercritical fluid extractions, pressurized solvent, enzymatic extraction, negative pressure extraction, and high-pressure homogenization (Sarker et al.,2024). Although Sorrenti et al.

(2023) mentions that traditional extraction methods are still the main approach for obtaining bioactive compounds.

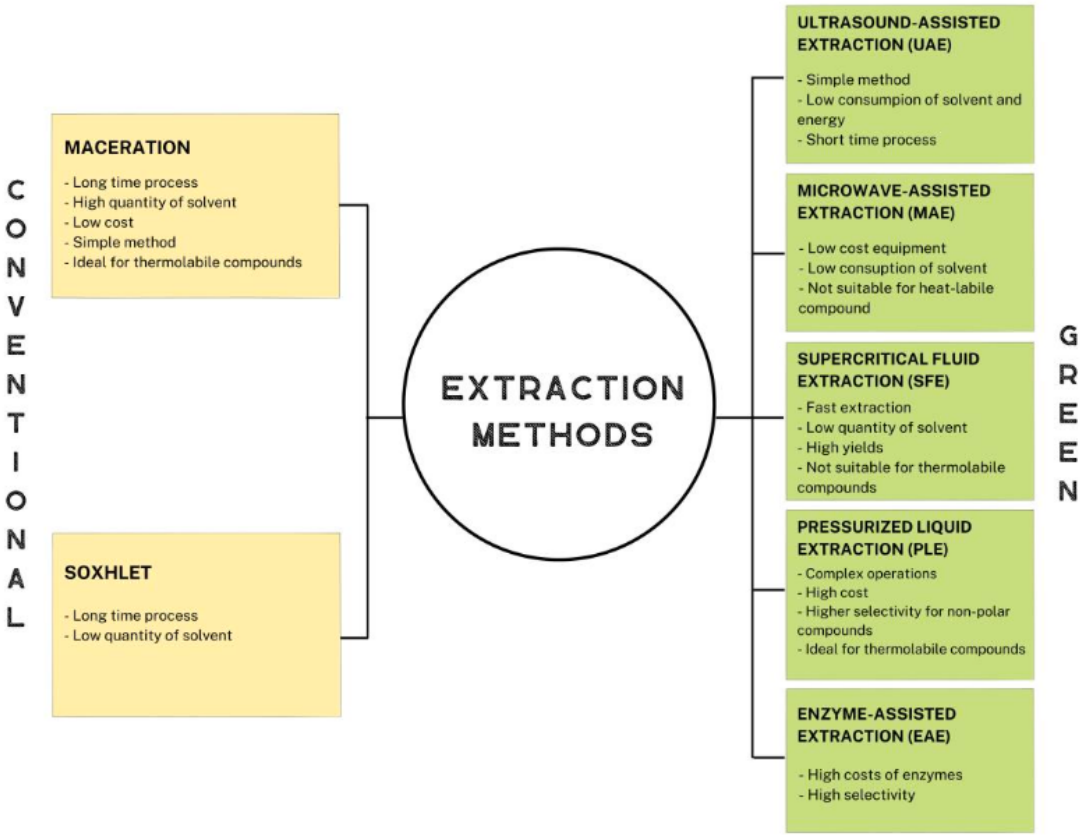


Figure 1: Advantages and limitations of conventional and green extraction methods (Sorrenti et al.,2023)

Among the many contributors to agri-food waste, the wine-industry stands out due to the vast quantities of byproducts it generates. Therefore, it is worth to explore how wine production waste, particularly grape pomace can be effectively valorized.

2.2. Wine industry waste valorization

Wine is among the most widely available alcoholic beverages globally, with the winemaking and juice industries processing approximately 44 million tons of grapes each season. In total, global grape production reaches around 77.8 million tons annually**,** making grapes one of the most extensively cultivated fruit crops worldwide. On average, every ton of wine produced generates around 200 kg of grape pomace, representing the industry's main waste component (Papaioannou et al.,2022). Leading wine-producing countries in Europe, such as Italy, France, and Spain**,** generate up to a million tons of solid residues from vinification and several million cubic meters of wastewater annually. The proper management

of these byproducts is crucial due to its significant environmental and economic implications (Sorrenti et al.,2023).

Winemaking techniques vary widely, ranging from large-scale industrial methods to small-scale artisanal approaches that continue to uphold traditional practices. **Figure 2** shows a summarized flow diagram of the vinification process highlighting the main byproducts, being grape pomace, wine lees, fining sediments, tartaric sediments and filtration residues (Salazar et al.,2018).

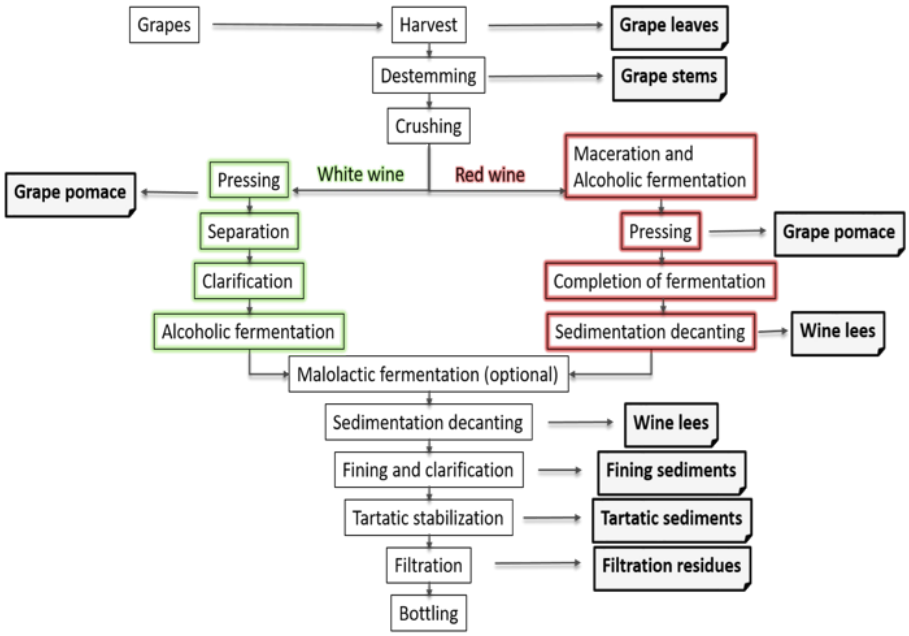


Figure 2: Global wine making process and main byproducts (Salazar et al.,2018)

The nature, as well as the physical and chemical properties of the final residues, are determined by the specific winemaking technique used. As mentioned previously among the solid residues, grape pomace is the most abundant waste product. It consists of skins, seeds, pulp, and stalks that remain after the grape pressing process (Salazar et al., 2018). Grape pomace is particularly rich in bioactive compounds such as anthocyanins, procyanidins, flavonoids, and stilbenes**,** which exhibit significant biological properties (Papaioannou et al.,2022). Apart from the employed wine making process, variations in the composition occur based on the diverse grape varieties and agricultural conditions of growth. Grape pomace derived from white and rosé wine being removed before alcoholic fermentation thus containing fermentable sugars is contrastable from the one derived from red wine. The proportion of seeds in this byproduct varies due to the same reasons, typically ranging between 38% and 52% of the dry matter**.** This component deserves special attention because it accounts for approximately 70% of the total

extractable polyphenolic compounds from grape pomace. Grape seeds are composed of up to 40% fiber, 11% proteins, 20% essential oil, and 8% polyphenolic compounds, for example tannins along with other components such as carbohydrates and minerals. The predominant polyphenols present in grape seeds belong to the flavonoid group**,** mainly flavan-3-ols, proanthocyanidin oligomers and polymers**.** The high concentration of phenolic compounds in grape seeds results from their limited extraction during the pressing stage of winemaking (Salazar et al., 2018).

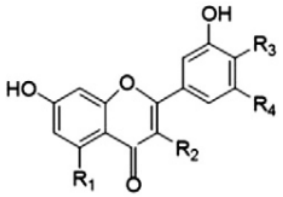
It is estimated that 70% of the biophenols originally present in grapes are retained in the byproducts generated from wine and juice production, making them a cost-effective organic source for extraction and recovery. These compounds are highly valued by experts from the pharmaceutical, cosmetic and food sectors. Moreover, there is a possibility to reuse wine industry wastes as soil fertilizers, distilled beverages, building construction materials, energy sources and livestock feed (Papaioannou et al.,2022; Salazar et al.,2018; Sorrenti et al.,2023; Devesa-Rey et al., 2011; Bustamante et al.,2008). As an example, for phenol recovery Syed et al. (2016) studied the valorization of grape pomace with the fractionation of bioactive flavan-3-ols by membrane processing, testing nanofiltration membranes. Nonetheless numerous other traditional or innovative techniques have been explored regarding the extraction of polyphenolic compounds from grape byproducts with varying yields. Conventional methods**,** as solid-liquid extraction, thermal processing, and enzymatic treatment; and non-traditional approaches involving pulsed electric fields, ultrasound-assisted extraction, microwave-assisted techniques, high-voltage electrical discharges, pulsed ohmic heating, sub- and supercritical fluid extraction, and pressurized liquid extraction were assessed (Salazar et al., 2018).

2.3. Grape seed extract

As the prior chapter explored, within the spectrum of wine production byproducts, grape seeds are of certain interest, due to their rich polyphenolic content. Therefore, what follows, is reviewing the composition and potential applications of grape seed extract derived from these seeds.

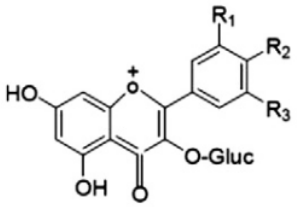
Clouatre et al. (2005) defined grape seed extract (GSE) as mixtures of catechin monomers, procyanidin oligomers and procyanidin polymers extracted from Vitis vinifera seeds. These polyphenolic phytochemicals can be categorized as reducing agents and hydrogen-donating antioxidants. The extract production method was specified by Kupina & Gafner (2016) the following way: „ The seeds of grapes, obtained as a byproduct from the juice or wine industry, are used fresh, or more commonly dried, to produce a liquid extract using a solvent

(e.g., water, or mixtures of water with ethanol or acetone), which is filtered, and may be subjected to further processing before it is typically spray-dried to obtain a dry extract containing high levels of naturally occurring grape seed phenolic compounds."



flavonols

	R_1	R_2	R_3	R_4
quercetin	OH	OH	OH	-
rutin	OH	O-Rutinose	OH	-
morin	OH	OH	-	OH
myricetin	OH	OH	OH	OH
fisetin	OH	OH	OH	-



anthocyanins

	R_1	R_2	R_3
peonidin-3-*O*-glucoside	OCH_3	OH	-
petunidin-3-*O*-glucoside	OH	OH	OCH_3
malvidin-3-*O*-glucoside	OCH_3	OH	OCH_3
cyaniding-3-*O*-glucoside	OH	OH	-
delphinidin-3-*O*-glucoside	OH	OH	OH

Figure 3: Chemical structures of some phenolic compounds from grape seeds (Xia et al., 2010; Perumalla & Hettiarachchy, 2011)

Polyphenol concentrations can range from 60% to 95% in GSEs. Monomeric flavan-3-ols recognized as (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-gallate esterified with a gallic acid, represent the phenolic fraction in the grape seed. These flavonoids show widespread distribution and notable chain extensions occur to produce polymers such as procyanidins (Clouatre et al., 2005). Perumalla & Hettiarachchy (2011) found that standardized grape seed extracts can contain 74 to 78% oligomeric proanthocyanidins and less than approximately 6% of free flavanol monomers on a dry weight basis. **Figure 3** shows the chemical structures of some phenolic compounds present in GSE. The presence and structural position of functional

groups -OH and -OCH_3 determine the antioxidant activity of the flavanoid molecule. While addition of -OH groups to the flavanoid nucleus enhances, their substitution by -OCH_3 groups reduces the antioxidant capacity. Another contributing factor is the polymerization degree of the procyanidins, as the higher it is, the higher is the antioxidant activity. It has been concluded that the antioxidant potential of GSE can be as much as 20-50 times broader than other well-known antioxidants such as vitamin C and vitamin E (Perumalla & Hettiarachchy, 2011; Gupta et al., 2020).

Naturally occurring bioactive compounds can be organized into four classes, being macronutrients, micronutrients, phytonutrients and gut microbiome regulators. These compounds have positive effects on human health. Amongst these classes, phytonutrients are categorized as natural compounds in plant foods such as vegetables, fruits, whole grain products, nuts, and legumes. Phenolic compounds in grape seed extract fall into the phytonutrient group (Kussmann et al.,2023). They are linked with antioxidant, cardioprotective, anticancer, anti-inflammation, antiaging and antimicrobial properties (Xia et al.,2010).

Antioxidative characteristics of phenolic compounds can be attributed to their metal chelating and free radical scavenging properties as well as their effects on cell signaling pathways and on gene expression. The antioxidant capacity of different phenolics has been studied and compared, as an example Soobratteea et al. (2005) found the order: procyanidin dimer, flavanol, flavonol, hydroxycinnamic acids and simple phenolic acids, from most to least antioxidative. However, it is likely that antioxidant activity of a sample is a combined effect of various compounds rather than a single composition (Xia et al., 2010). Queseda et al. (2009) found that natural proanthocyanidins improve dyslipidemia associated with high-fat diet, by repressing lipogenesis and VLDL (very-low-density lipoprotein) assembly in the liver. Hemmati et al. (2014) evaluated the effect of GSE 2% herbal cream on human skin lesions and observed the difference of complete wound repair on day 8 for the treatment group, while on day 14 for the placebo group. In conclusion proanthocyanidins in GSE were deemed to trigger the release of vascular endothelial growth factor, and the topical application caused wound contraction and closure with increased amount of connective tissue and improved cellular structure of wound. Costa et al. (2015) assessed the effects of an oral supplement containing (per tablet) marine protein (105 mg), vitamin C (27 mg), grape seed extract (13.75 mg), zinc (2 mg), and tomato extract (14.38 mg) and found improvements of skin aging in men. Gupta et al. (2020) found from sources that GSE is able to improve antioxidant defenses, such as preventing ischemia / reperfusion induced and carbon tetrachloride induced liver injury and it is capable to reduce the risk of cardiovascular diseases by inhibiting the oxidation of LDL (low-density

lipoprotein) improving endothelial function, inhibition of platelet aggregation, reducing inflammation, lowering blood pressure and prevent cell senescence by activating novel proteins. Ghanbari et al. (2024) investigated the effects of 520 mg GSE/ day for 2 months on metabolic factors, blood pressure and steatosis severity in patients with non-alcoholic fatty liver disease and concluded that GSE can be considered one of the appropriate strategies for controlling insulin resistance, hyperlipidemia, hypertension and hepatic steatosis. Seo et al. (2025) conducted the bioconversion of wine grape seed flour extract and whey with kefir lactic acid bacteria and found it a useful strategy to establish healthy gut microbiota and prevent sarcopenia.

The previous paragraph lists a few of the several studies carried out throughout the years exploring the health impacts of GSE. It has been observed that phenolic compounds from grape seed displayed a higher phenolic content and antioxidant capacity by in vitro physiological procedure such as digestive enzymatic extraction than by chemical procedure, which justifies the presence of the wide range of dietary supplements on the global market containing GSE (Li et al., 2008; Xia et al., 2010). In the study of Yamakoshi et al. (2002) conducted with rats the no-observed-adverse effect level (NOAEL) of GSE translated to 1410 mg/kg body weight in males and 1501 mg/kg body weight/day in females. The definition of functional foods according to the FAO Terminology Portal is as follows: „A foodstuff that provides a health benefit beyond basic nutrition, demonstrating specific health or medical benefits, including the prevention and treatment of disease." and „Processed foods containing ingredients that aid specific bodily functions in addition to being nutritious." (Internet 2). Based on this characterization GSE can be considered as a functional food ingredient.

In the industry GSE can be used also for the preservation of food products due to its natural antimicrobial and antioxidant properties instead of other synthetic antioxidants like tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), the dose of which are strictly regulated. For example, the preserving effect of GSE has been evaluated in beef and chicken meat products and sunflower oil (Patel 2015; Ma & Zhang 2017). In the Substances Added to Food database of the Food and Drug Administration (FDA) GSE is marked as a flavoring agent or adjuvant (Internet 3). Proanthocyanidins in grape seed extracts have a red color and astringent taste, therefore in higher concentrations they can affect the sensory and color properties of a product (Ma & Zhang, 2017). Davidov-Pardo et al. (2012) investigated the sensory and consumer perception of the addition of bulk and microencapsulated GSE in cookies. The addition of GSE resulted in darker brown cookies and microencapsulation partially masked this darker color. The cookies

with the microencapsulated GSE showed a significantly higher antioxidant activity, indicating low heat degradation. Enriched cookies were regarded astringent and with aromas and flavors similar to whole grains flours. After the assessment, nearly 60% of consumers stated that they were willing to purchase cookies enriched with antioxidants.

As the demand for grape seed extract has grown, so too have concerns over authenticity and purity of commercial products. This leads to further inquiry about the significant issue of food adulteration in the modern food industry.

2.4. Adulteration in the food industry

Food adulteration is a long-term global problem affecting the food industry. The Grocery Manufacturers Association estimates that fraud may cost the global food industry between $10 billion and $15 billion per year, affecting approximately 10% of all commercially sold food products (Johnson, 2014). Overall, the two main distinguishable categories include incidental and intentional adulteration. The former is attributable to lack of hygienic practice throughout production and transportation. Accidental adulterants can be pesticides residues, dropping of rodents, larvae in foods, and even metallic contamination with arsenic lead and mercury can occur. Unintentional adulterants also involve pests such as rodents and insects that trespass food, producing impurity in the form of excreta, bodily secretions and spoilage through microorganisms. Conversely the food item is deliberately adulterated when there is inclusion of inferior substances having properties similar to the foods in which they are added. They are thus difficult to detect (Choudhary et al., 2020). Narayan (2014) summarized the possible causes of food adulteration into the following six points:

1. The demand is more than the supply in the market,
2. To come at par with the market competitors, lowering the cost of production is needed,
3. Greed for increased profit margins,
4. Many individuals are unable to afford food items with their original constituents,
5. Lack of trained manpower with outdated food processing techniques, and
6. No knowledge about the disease outbreaks caused due to adulterated food products.

According to FDA (Food and Drug Administration) economically motivated adulteration (EMA) occurs when someone intentionally leaves out, takes out, or substitutes a valuable ingredient or part of a food. EMA also occurs when someone adds a substance to a food to make it appear better or of greater value (Internet 1). **Table 1** exemplifies some food and beverage items with their characteristic adulterants and possible purposes of adulteration.

Table 1: Different food items and their adulterants (after Choudhary et al., 2020)

Food and drink items	Adulterant/extraneous substances	Purpose	Type of adulteration
Ghee	Vanaspati, anatta, and oleomargarine	To make it more yellow	Deliberate
Milk	Water, skim milk	To increase volume	Deliberate
Condensed milk	Paneer, khoya	To give rich texture	Deliberate
Butter	Vegetable oil, anatta, banana, oleomargarine	To increase volume and make it yellowish	Deliberate
Tea leaves	Black/Bengal gram dal husk with colour	To add color	Deliberate
Red wine	Juice of bilberries	To attract/produce deep blue precipitate with lead acetate	Deliberate
Black pepper	Papaya seed	To add bulk	Deliberate
Green chilies and peas	Malachite green	To give bright glowing green colour	Deliberate
Chilies powder	Brick powder	To increase weight	Deliberate
Sugar	Chalk powder	To increase amount	Deliberate
Oils	Rancid oil	To increase volume	Deliberate
Coriander powder	Cow dung powder	To increase amount	Deliberate
Common salt	White powdered stone, chalk	To increase amount	Deliberate
Coffee	Chicory, roasted barley powder, tamarind seeds	To add bulk and colour	Deliberate
Honey	Molasses, cane sugar	To increase volume	Deliberate
Wheat	Ergot (poisonous fungus)	To increase weight	Deliberate

Fraud has notable economic consequences and can lead to public health risk events. In 1981, a batch of industrial rapeseed oil was illegally contaminated with aniline, and it was then sold as olive oil to street traders across Spain. 20000 persons got affected, approximately 300 died shortly after the onset of the disease and a larger number developed chronic disease (Gelpí et al.,2002). In 2008 melamine, a chemical used in plastics, had been found in domestic dairy products in China and many people, especially young children, were experiencing adverse health impacts including death. Melamine is nitrogen-rich and was added to watered-down milk to fool food quality inspectors, who often use nitrogen levels to measure protein levels. The perceptions and consumption of fluid milk, yogurt, milk powder and ice cream had plummeted after these events (Qiao et al., 2010).

Countermeasures against food frauds are identified in the ambit of food quality standards. The most known of these standards are:

- The Global Standard for Food Safety (GSFS) by the British Retail Consortium (BRC)
- The International Featured Standard (IFS) Food, by the IFS
- The ISO 22000 norm, by the ISO
- The Food Safety System Certification (FSSC) 22000
- The Safe Quality Food (SQF) by the SQF Institute, USA
- The Roundtable on Sustainable Palm Oil (RSPO) certification scheme, by the homonym organization.

The International Organization for Standardization (ISO), in particular ISO 9001:2015 (ISO 9015), have been elaborated with the aim of giving a strong adherence to the food and beverage sector (Fiorino et al. 2019). A model of food integrity management and mitigation was developed by Manning (2016), shown on **Figure 4**. It has three key constructs (1.) food integrity which is characterized as four elements: product integrity, process integrity, people integrity and data integrity, (2.) drivers of supply chain of food fraud, and (3.) example mitigation measures which are differentiated as operating at three levels: organizational, supply chain and global measures. Product and process verification is of main importance. Product integrity can be verified with the use of rapid and reliable sensor, spectroscopic and chromatographic techniques combined with the use of multivariate and multiway chemometrics, if the tests have been developed and validated (Manning, 2016).

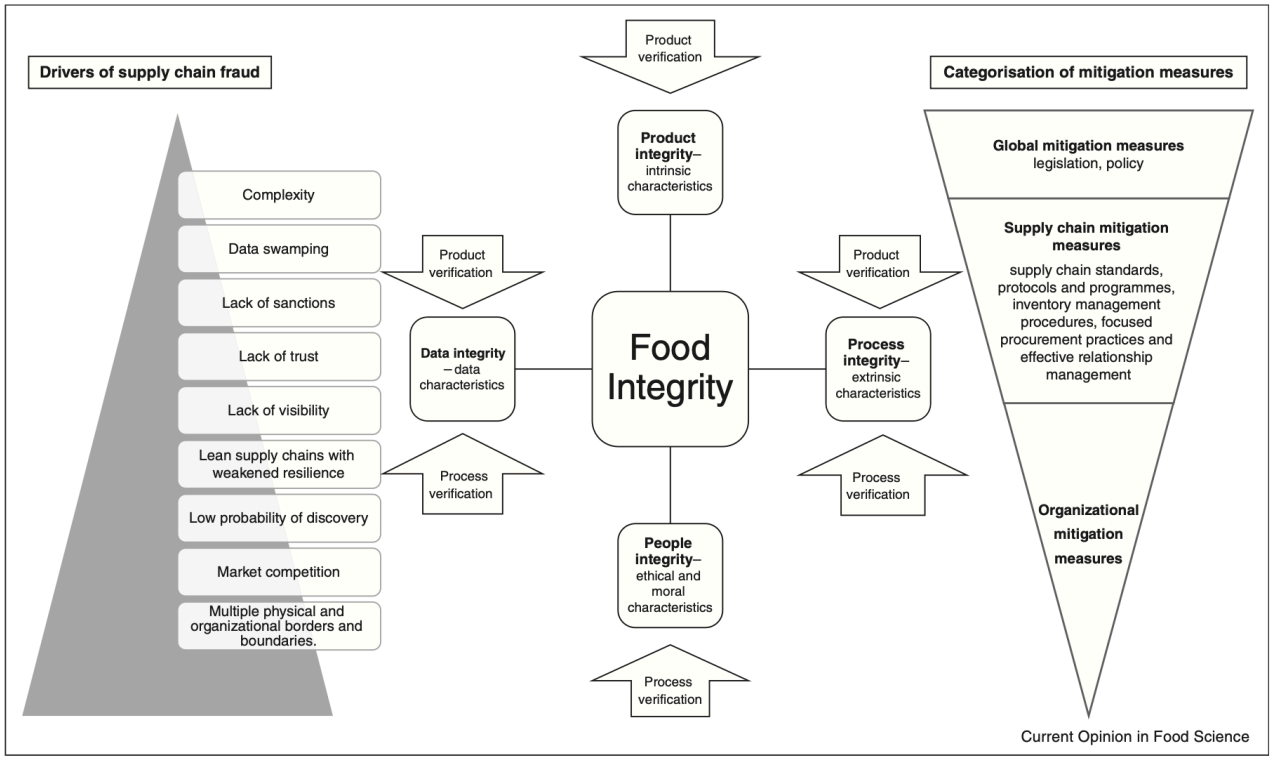


Figure 4: Model of food integrity management and mitigation (Manning, 2016)

While food adulteration is a widespread problem across many commodities, GSE presents unique challenges due to the high similarity of potential adulterants. Accordingly, the following chapter narrows the focus to the specific issue of GSE adulteration.

2.5. Adulteration of grape seed extract

Due to the wide range of scientific findings regarding health benefits, GSE is one of the most broadly used botanical extracts. Its popularity amongst consumers rocketed in the early 2000s, with its primary application form being dietary/food supplements. While companies manufacture from purchased grape seeds intentional adulteration can take place in order to artificially increase the concentration in total phenols and increase the volume by using other proanthocyanidin rich substances. The commonly applied analytical methods are not specific enough to distinguish between grapeseed proanthocyanidins and proanthocyanidins from other plant extracts, therefore adulteration can go undetected throughout the food chain (Kupina & Gafner, 2016). Villani et al. (2015) profiled 21 commercial GSE containing products chemically and compared against authenticated GSE, peanut skin extract (PSE) and pine bark extract (PBE). HPLC/UV/MS, standard total polyphenol content (TPC) and antioxidant capacity measurements were used for each sample. Nine products were adulterated and found to contain PSE, 6 of which was composed of PSE entirely.

Known adulterants of GSE are PSE (Arachis hypogaea) PBE (Pinus spp.,Pinaceae) ,green tea extract (GTE, Camellia sinensin, Theaceae) and other proanthocyanidin (PAC) rich extracts like propelargonidin-containing extracts, for example: raspberry (Rubus idaeus subsp. idaeus or R. idaeus subsp. strigosus, Rosaceae), strawberry (Fragaria vesca or F. virginiana, Rosaceae), common beans (Phaseolus vulgaris, Fabaceae), almond (Prunus dulcis, Rosaceae), cinnamon (Cinnamomum verum, Lauraceae), buckwheat (Fagopyrum esculentum, Polygonaceae), mountain ash (Sorbus aucuparia, Rosaceae) berries, hops (Humulus lupulus, Cannabaceae) (Kupina & Gafner, 2016).

Commercial GSE has been found to contain mainly B-type PACs (largely B2, B1, B3, B4 from highest to lowest concentrations), therefore the adulteration with B-type procyanidin rich materials presents a higher challenge. **Table 2** lists the PAC characteristics of grape seed, and some of its possible low-cost adulterants. PSE mostly consists of A-type PACs; GTE is primarily composed of catechin, the main flavan-3ol being epigallocatechin gallate (EGCG); while PBE primarily has B-type PACs, and according to Weber et. al. (2007) in general also uniquely contains taxifolin-gallate (Kupina et al., 2019). In line with the findings of Villani et. al. (2015) evaluating thin layer chromatography (TLC), pine bark extract contains more dilute content of catechin, and PAC dimer compared to GSE. **Figure 5** represents the structures for (epi)catechin, A-type proanthocyanidins, and B-type proanthocyanidins.

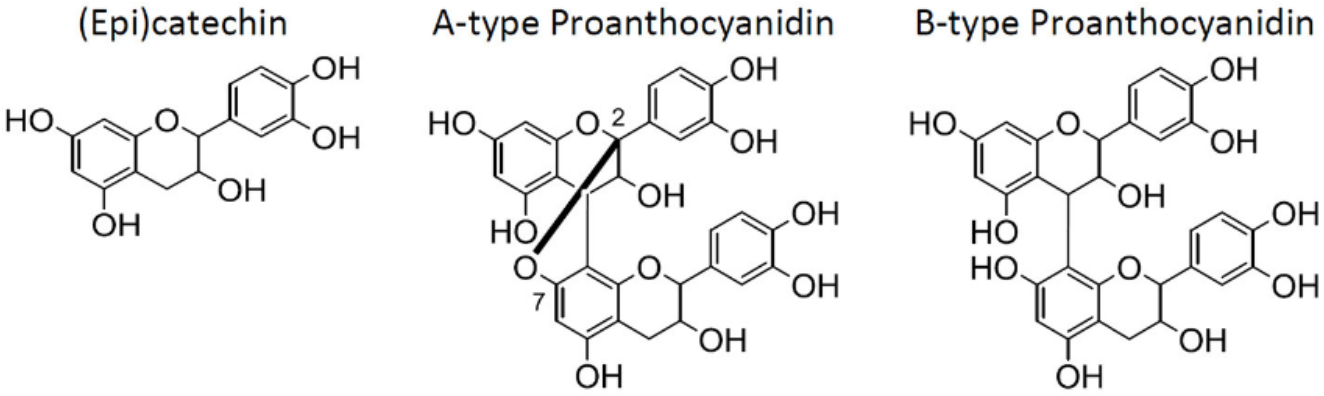


Figure 5: The structures for (epi)catechin, A-type proanthocyanidins, and B-type proanthocyanidins (Sica et al.,2018)

Table 2: Proanthocyanidin characteristics of grape seed and a few low-cost adulterants (after Kupina et al., 2019)

Ingredient	Monomer(s)	Galloylation	PAC-type	Average degree of polymerization
Grape seed	Catechin, epicatechin	Yes	B-type	2-12
Peanut skin	Catechin, epicatechin	No	A-type, B-type	1-9
Maritime pine bark	Catechin, epicatechin, epigallocatechin, gallocatechin	Yes	B-type	3-7
Masson pine bark	Catechin, epicatechin, epigallocatechin, gallocatechin	Yes	A-type, B-type	No data
Green tea leaf	Catechin, epicatechin, epiafzelechin, epigallocatechin, gallocatechin	Yes	B-type	1-1.1

Adulteration of GSE is a significant problem, posing both safety concerns and economic impacts. Utilizing PSE this way risks severe allergic reactions for consumers with peanut allergies. GTE can interfere with iron absorption. Overall tampering with PSE, PBE and GTE can reduce the bioavailability of B-type procyanidins which are mainly responsible for the beneficial cardiovascular properties of GSE (Fan, 2016; Kupina & Gafner, 2016). Falsifying materials are typically less expensive than grape seeds, thus companies producing authentic GSE lose sales, due to consumers making a price-oriented purchasing decision. Furthermore, manufacturers who purchase already adulterated extracts and conduct analysis which can be fooled or rely on inferior quality control procedures are at a risk of regulatory actions and are being defrauded (Kupina & Gafner, 2016).

To tackle the problem of adulteration, suitable analytical techniques must be employed. This leads to the final part of this section providing an overview of the main methods used in the food industry to identify adulteration and evaluate product authenticity, with a focus on chromatography and infrared spectroscopy.

2.6. Analytical techniques in the food industry to identify adulteration

There is a broad range of analytical methods to inspect foodstuff, test authenticity, detect adulteration and fraud. Hong et al. (2017) collected the main techniques used reported from 2005 until 2015 regarding various food categories, shown on **Figure 6**.

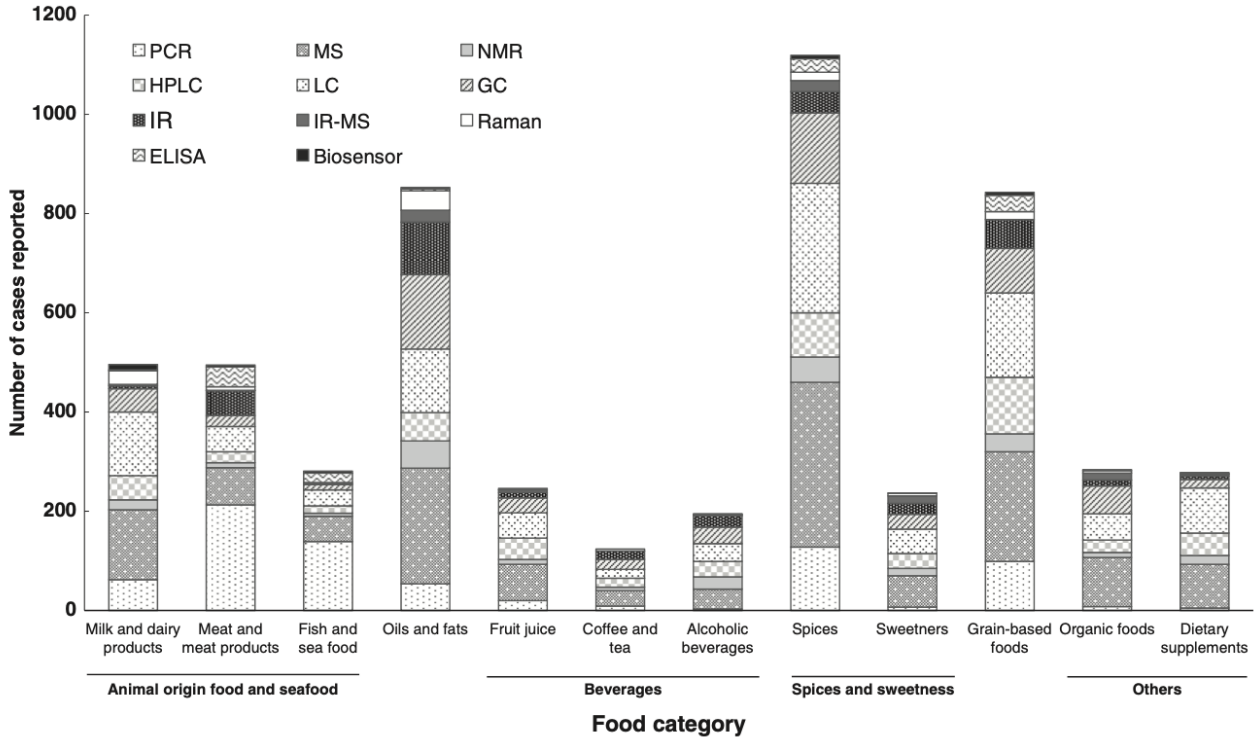


Figure 6: Reported adulteration detection techniques for different food categories (Hong et al., 2017)

These include chromatographic analysis such as thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC); mass spectrometry (MS)-based methods, like liquid chromatography(LC)-MS/MS, GC/MSD; electrophoretic methods, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR); spectroscopic methods, for example infrared (IR), near infrared (NIR), mid-infrared (MIR), Fourier transform infrared

(FTIR), Raman, nuclear magnetic resonance (NMR), site-specific natural isotope fractionation (SNIF)-NMR, inductively coupled plasma-atomic emission spectrometer (ICP-AES); and immunoassays, namely enzyme-linked immunosorbent assay (ELISA) (Hong et al., 2017). Electrical sensors like electronic noses and tongues are also deserved to be mentioned amongst these techniques when it comes to the chemical determination of food adulterants by imitating human sense (Anagaw et al., 2024; Yong et al., 2020; Ellis et al.,2012). Moreover, other optical detection methods include computer vision and spectral imaging, including Raman imaging, hyperspectral imaging (HSI), laser induced breakdown spectroscopy (LIBS), terahertz spectroscopy and terahertz imaging, fluorescence spectroscopy (FS) and photoacoustic spectroscopy (PAS) technique (Yong et al., 2020).

Chromatography refers to a group of analytical techniques employed to separate chemical constituents within a mixture, including food substances, primarily based on their polarity or boiling point. These methods rely on the differential distribution of analytes between two phases: a stationary phase and a mobile phase. Classification of chromatographic techniques typically depends on the physical and chemical nature of these phases. In practice, the stationary phase (solid) is housed within a column, while the mobile phase(liquid) is actively passed through it. Compounds with weaker interactions with the stationary phase, lower boiling points, and shorter retention times tend to move through the column more quickly, reaching the detector earlier than those with stronger affinities. GC and LC are two of the most wildly used classifications of chromatography. While LC can analyze both polar and non-polar solvents directly and easily without derivatization, it is needed for GC during sample preparation to change boiling points. HPLC fingerprints are used to compare the similarity of various food stuff and apply to calculate correlation parameters. Nowadays LC systems are transitioning from conventional HPLC to UHPLC (ultra-high-performance LC), which requires decreased sample volumes and mobile phases, while enhancing resolution, sensitivity, and peak capacity, primarily using smaller particle sizes (Anagaw et al., 2024). Chromatographic methods can be called traditional analysis techniques for the detection of food adulteration and can accurately measure relevant chemical components and adulterant concentrations. However, most of these applications require sample pretreatment and they also destroy samples during operation. Furthermore, conducting these measurements is an expensive and complex process, which makes them non-available for large scale on-line utilization (Yong et al., 2020). Additionally, in an environmental point of view solvents generally applied during LC methods such as acetonitrile, methanol and chloroform can raise concerns due to their volatility, toxicity and

(excluding chloroform) flammability. It is also estimated that a single liquid chromatograph can generate 1 to 1.5 liters of liquid waste per day (Płotka et al., 2013).

Infrared spectroscopy is an analysis of absorption spectra. It can be applied as a rapid non-destructive analysis technique which can obtain information without damaging a sample's integrity. During operation the sample is irradiated by infrared light and its molecules absorb infrared radiation of a certain frequency which causes the transition of the vibrational and rotational energy levels of the molecules. The infrared absorption spectra of the sample can be obtained by detecting the absorption of infrared rays. The application of this method is possible to gaseous, solid and liquid samples as well. For the analytical inspection of foodstuff mostly near-infrared (NIR) and mid-infrared (MIR) spectroscopy are implemented. MIR region is in the wavenumber range of 2500-12500 nm and mainly concerned with the vibrational rotation of molecules. Fourier transform infrared (FTIR) determines the interference pattern of a sample and is formed by combining the Fourier transform technique in mathematics with computer technique and infrared spectroscopy (Yong et al., 2020). NIR spectroscopy (NIRS) operates in the near infrared region of the electromagnetic spectrum between 750 and 2500 nm. When the light interacts with the sample's molecules, the bond of these molecules vibrates at different frequencies, depending on the type of bond; CH, NH, OH and SH vibration bonds being the most prevalent in this region. These bond vibrations determine the shape of the spectra of a given sample. The obtained spectrum is characterized by the overlap of these bonds in broad bands. This method can be called low-selective and captures only the overtones and combination tones of vibrations derived from the functional bonds. Assessment and quantification of the basic chemical profile is the main use of NIR spectroscopy, however uniquely the NIR spectrum also reflects the physical attributes of a sample (Cozzolino, 2021). Overall NIRS has several advantages, including minimal sample preparation, rapid detection, high efficiency, low cost and large-scale applicability (Yong et al., 2020). Portable NIR instruments also enable on-site analysis requiring a few seconds, adaptable across the entire chain, from production and sourcing of raw materials, through process monitoring to quality control of final products (Folli et al.,2022; Ellis et al., 2012). Compared to MIR, NIR light penetrates much deeper into an intact food matrix, even when it is enclosed into packaging materials transparent to NIR. Furthermore, analysis can be more routine, because water absorbance in the NIR is weaker than in the MIR (Ellis et al., 2012). Considering these attributes and the fact that this technique doesn't require the use of toxic solvents or reagents, it can be called a green analytical method.

For analysis of specific adulterants, for example the quantification of a chemical compound in a food sample with HPLC, which has a known retention time, only the creation of standard curves for absolute quantification is required without the use of intricate data processing. On the other hand, the spectra obtained after NIRS are highly complex, due to the simultaneous detection of all chemicals and overtones appearing as highly overlapped peaks. The relevant information from these spectral fingerprints needs to be extracted using chemometric methods, which aim to build a multivariate model, characterizing the system under study. The main steps of chemometrics are experimental design and data collecting, preprocessing, exploratory data analysis, model building-calibration and finally model validation. Common chemometric terms and strategies are detailed in **Table 3 and 4** (Ellis et al., 2012). A NIR spectrometer can be used routinely only after calibration and extensive testing to verify their predictive ability or to track any deviations regarding a light source, sensor or electronics (Cozzolino, 2021).

Gardana et al. (2018) validated a FT-NIRS method with principal component scores for the prediction of bilberry extract adulteration and partial least squares regression (PLSR) model for total anthocyanin evaluation and obtained results showing the potential of this technique in the discrimination of genuine bilberry extracts from the ones adulterated with anthocyanins extracted from other berries. Walkowiak et al. (2019) found that Attenuated Total Reflectance-FTIR spectroscopy combined with chemometric modeling using interval partial least squares-linear discriminant analysis (PLS-DA) is an efficient screening method for the preliminary evaluation of adulteration of Ginkgo biloba supplements with kaempferol, rutin or quercetin. Wang et al. (2022) used NIRS combined with chemometrics to detect adulteration in quinoa flour, applying principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) during the initial exploration, then building PLSR model with the spectral data. The used method was proven to be reliable for the quantification and detection of wheat flour adulterants. Shawky et al. (2020) applied NIRS combined with multivariate data analysis. Soft Independent Modeling of Class Analogy (SIMCA) was used for saffron authentication and PLS discriminant analysis effectively discriminated authentic and intentionally adultered samples with as low as 10 mg/g plant adulterants including exhaustively extracted saffron stigmas and saffron stamens. PLSR models reliably predicted the quantitative amount of each adulterant in saffron samples, showing great potential. These previously presented studies are few of the several which utilize NIRS combined with chemometrics to detect adulteration of samples with plant origin. However, amongst the published scholarly articles there is a very limited number which focuses on the proanthocyanidin based adulteration in grape seed extracts

and even less if any exploring NIRS-based methods combined with chemometrics for the quantification of specific GSE adulterants.

Table 3: Definition of common chemometric terms (After Ellis et al., 2012)

Multivariate data	Spectroscopic data comprising many variables (e.g., absorbances at specific wavenumbers for FT–IR, or ion counts at specific m/z for MS) collected on the samples. Often referred to as the X-data or input data.
Metadata	Data about the data. This information is used in classification or quantification modelling, often referred to as the Y-data or the output data. These are the traits one is interested in, e.g., country or species of origin, level of adulteration, etc.
Pre-processing	Methods used to go from raw data to clean data ready for analysis. This may include removing baseline artefacts, peak-picking or alignment.
Pre-treatment	Transforming the pre-processed data to make them suitable for analysis. This typically includes normalization, scaling, transformations and removing any outliers (unusual, atypical samples) in the data.
Unsupervised learning	Analysis conducted on only the X-data with the goal to generate clusters from these input data. This process is often referred to as dimensionality reduction or simplification. After clustering ordination (2D or 3D) plots or dendrograms (tree-like diagrams) are used to visualize the clusters.
Supervised learning	Analysis conducted on both the spectroscopic X-data and Y-data. This process uses some mathematical transformation to associate the X-data with the target trait (Y-data). This process is often achieved by reducing the error between the model's output prediction and the actual known target trait. It is therefore essential that the Y-data must be unequivocally known during model construction.

Table 3: Definition of common chemometric terms (After Ellis et al., 2012)

Classification modelling	The goal is to classify samples into groups. This may be for different countries of origin. For example, if there are three countries then the Y-data are encoded such that a '1' is placed in the correct country and '0s' elsewhere, so that country A is represented by '100', B by '010' and the third country C as '001'.
Quantitative modelling	The goal here is to quantify the trait of interest. This is usually the level of adulterant/contaminant, and the Y-data is simply the level of the trait of interest.

Table 4: Employable chemometric methods and strategies for the analysis of spectroscopic data (After Ellis et al., 2012)

Modelling method	**Model type**	**Unsupervised or supervised**	**Comments**
Principal components analysis (PCA)	Exploratory	Unsupervised	Very old method used to explore the data to look for any obvious clusters and for the detection of outliers. These are viewed in ordination plots of the PC scores. Relevant chemical information can be extracted from the loadings matrix.
Hierarchical cluster analysis (HCA)	Exploratory/ Summative	Unsupervised	Generates a dendrogram which is a tree-like structure where the leaves are the samples, and the branches shows their relationships
Self-organizing (feature) maps (SO(F)M)	Exploratory	Unsupervised	Kohonen neural network based on unsupervised learning. Output nodes cluster similar samples together

Table 4: Employable chemometric methods and strategies for the analysis of spectroscopic data (After Ellis et al., 2012)

Modelling method	Model type	Unsupervised or supervised	Comments
Partial least squares regression (PLSR)	Quantitative	Supervised	Multivariate linear regression method
Discriminant analysis (DA)	Classification	Supervised	Series of algorithms that perform classification resulting in scores plots where the goal is to separate clusters according to group classifications. Includes linear discriminant analysis (LDA), discriminant function analysis (DFA), factorial discriminant analysis and the very popular PLS-discriminant analysis (PLS-DA)
Artificial neural networks (ANNs)	Quantitative or classification	Supervised	Multilayer perceptrons (MLPs) are powerful approaches that can map non-linear functions from X-data to Y-data.
Evolutionary-based analysis	Quantitative or classification	Supervised	Series of algorithms to perform variable selection. Based on Darwinian concepts. Common methods include genetic algorithms (GAs) and genetic programming (GP)
Classification and regression trees (CART)	Classification	Supervised	Results in a tree where the leaves are categories of samples and the branches are decision boundaries
Random forests	Classification	Supervised	An ensemble classification algorithm that uses many CART solutions to allow more robust predictions.

3. MATERIALS AND METHODS

This section details the materials used, and the experimental protocols applied, including sample preparation, chemical analysis, spectral acquisition, and statistical modeling in line with the defined objectives above.

3.1. Samples and their preparation

Authentic powdered extracts of grape seed (GSE), pine bark (PBE), peanut skin (PSE), and green tea (GTE) were sourced from Xi'an Rongsheng Biotechnology Co., Ltd. (Shaanxi, Cina). The used materials were authenticated by HPLC method, defined by Villani et al. (2015). GSE was mixed with PBE, GTE and PSE to formulate singular mixtures, where only a single additive was used (PBMIX, GTMIX, PSMIX), dual mixtures (PBMIX + GTMIX, PBMIX + PSMIX, GTMIX + PBMIX) where two additives, and three - component mixtures (PBMIX + GTMIX +PSMIX) where all three adulterants were mixed to GSE simultaneously. Ten additive levels (0.5, 1, 1.5, 2, 3, 5, 7, 9, 11, 13 *w*/*w*%) were prepared this way, with the individual concentrations of added extracts in dual mixtures halved (e.g., 0.25–0.25 *w*/*w*% of PB–GT for level 1, 0.5–0.5 *w*/*w*% for level 2, etc.), and in case of the three - component mixtures the concentrations were divided into three parts (e.g., 0.17–0.17-0.17 *w*/*w*% of PB–GT-PS for level 1, 0.33–0.33-0.33 *w*/*w*% for level 2, etc.). The concentration range was chosen to reflect common industrial practices involving the adulteration and fortification of these compounds. Additionally, samples containing only the pure compounds (GSE, PBE, GTE, PSE) were prepared. All samples were meticulously homogenized in plastic sample holders. Pure GSE samples were prepared in 10 replicates, while all other samples were in triplicates, resulting in a total of 267 samples. (Lukacs et al., 2024).

3.2. Antioxidant Capacity Determination

To assess antioxidant activity (AA), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was employed, following the method described by Brand-Williams (1995).

A total of 0.5 g of GTE, GSE, PBE and PSE samples were dissolved in 5 mL of methanol and stirred for 30 minutes. Subsequently 134 μL of each sample solution was added in a cuvette containing 3.9 mL of a $6 * 10^{-5}$ M methanol solution of DPPH. The cuvette was incubated in the dark for 40 min at room temperature. The bleaching of DPPH was measured at 515 nm using a UV–6300PC Double Beam Spectrophotometer (VWR International S.r.l., Italy) and

recorded as *Asample*. A blank experiment was prepared using the same procedure without the sample and recorded as *Ablank*. Antioxidant activity was expressed as the percentage of DPPH inhibition and calculated using the following formula:

$$\%\ inhibition\ of\ DPPH = \frac{(A_{blank} - A_{sample})}{A_{blank}}$$

Antioxidant activity was ultimately expressed as Trolox equivalent. The values were obtained using a calibration curve of Trolox standard solutions ranging from 60 to 600 μM, prepared following the same protocol as the samples. The results were reported as μmol of Trolox equivalent (TE) per gram of sample. Reagents were sourced from VWR International S.r.l., Milan, Italy (Lukacs et al., 2024).

3.3. Total Polyphenol Content Determination

Total polyphenol content (TPC) was determined by homogenizing GTE, GSE, PBE and PSE sample (1 g) in a methanolic solution (methanol/water 80/20 v/v). Each sample was centrifuged at 5000 rpm for 5 min and the supernatant was used to estimate TPC according to the Folin-Ciocalteu method (Singleton et al., 1999) by reading the absorbance at 760 nm. Results were expressed as mg gallic acid equivalents of g dry weight basis (DW, mg GA/g).

3.4. HPLC Method for Polyphenol Detection

HPLC analysis was performed using a Hewlett Packard HP-1100 series (Palo Alto, CA, USA) fitted with an auto sampler and a diode-array detector (DAD). The separation was achieved on a Poroshell 120 EC-C18 column (150 × 4.6 mm I.D., 4 μm particle size) (Agilent, Santa Clara, CA, USA). The column temperature was set at 25 °C, and the injection volume was 2 μL. The mobile phase was used in gradient mode. The elution was carried out using two solvent systems: 1% acetic acid in acetonitrile (v/v) as solvent A and 1% acetonitrile in water as solvent B, with a flow rate of 1.2 mL/min. HPLC/DAD analyses were conducted monitoring at 280, 310 and 350 nm. Phenolic compounds were identified by comparing retention time and UV absorption spectra with available standards. Quantification was achieved using calibration curves generated from external standards, constructed by plotting HPLC peak areas against known concentrations (mg/L). Final values were expressed in mg/g. Reagents were obtained from VWR International S.r.l., Italy (Lukacs et al., 2024).

3.5. NIR Spectral Acquisition

Spectral acquisition was carried out using a benchtop NIRS XDS instrument (Metrohm, Glostrup, Denmark) equipped with the RapidContent Analyzer module. The system utilizes a dispersive grating monochromator to record spectra within the 400–2500 nm wavelength range at 0.5 nm intervals. Diffuse reflectance signals were captured using a silicon (Si) detector for the 400–1099.5 nm range and a lead sulfide (PbS) detector for the 1100–2500 nm range. Sample spectra were acquired through a cuvette with an optical glass window. To reduce variability in light scattering, powdered samples were uniformly compacted by gently tapping the cuvette three times on a laboratory bench prior to measurement.

All spectra were recorded at room temperature (25 °C) using three consecutive scans, yielding a total of 801 readings. Temperature and humidity during spectral acquisition were monitored with a Voltcraft DL-121TH multi-data logger (Conrad Electronic, Berlin, Germany) to account for potential environmental influences (Lukacs et al., 2024).

3.6. Statistical methods

3.6.1 Univariate Statistical Comparison of the Chemical Results

A one-way ANOVA was conducted to determine significant differences in the TPC and AA values obtained from each raw extract. Prior to analysis, the suitability of the data for ANOVA was confirmed: homogeneity of variances was assessed using Levene's test, and normality was evaluated using the Shapiro - Wilk test. Skewness and kurtosis were also assessed. As a result, if ANOVA assumptions were met, pairwise comparisons among the extracts were performed using Tukey's HSD (Honestly Significant Difference) test. Pearson correlations between AA, TPC and HPLC values were also evaluated.

3.6.2 Exploratory Analysis of the NIR Spectroscopy Results

Raw spectra obtained from the benchtop spectrometer were initially examined to identify key absorbance regions and determine the most suitable spectral pretreatment. Various of these were applied to minimize baseline shifts and slope variations due to spectral noise and other unwanted variance. These included the Savitzky–Golay (SG) filter with a second-order polynomial and smoothing points ranging from 11 to 41, multiplicative scatter correction (MSC) and detrending (deTr). The raw spectra of extracts and extract mixtures were visualized also after the applied pretreatments to detect and align prominent absorbance peaks.

Principal Component Analysis (PCA) was applied to explore data structure, identify patterns, and detect outliers. The optimal pretreatment combinations were chosen based on observations of the raw spectra and PCA score plots and further refined during the supervised modeling phase. Preference was given to combinations that effectively minimized baseline distortions and curvature inconsistencies while enhancing chemically relevant absorbance features and regions. In parallel, specific wavelength intervals were selected for analysis, concentrating on regions deemed most informative during the exploratory phase. Wavelength segments exhibiting poor signal-to-noise ratios, abnormally high absorbance, or spectral artifacts related to the instrument or sensor characteristics were typically excluded (Lukacs et al., 2024).

3.6.3 Predictive Modelling of the NIR Spectroscopy Results

Partial Least Squares Regression (PLSR) was employed to model and predict the concentration of each extract (GSE, PBE, GTE, PSE), antioxidant activity, total polyphenol content, and the concentrations of specific phenolic compounds (gallic acid, catechin, epicatechin). Each PLSR model was individually optimized to achieve maximum predictive accuracy while avoiding overfitting and overly optimistic results. Cross-validation was performed using a leave-three-consecutive-out strategy, and the most promising models underwent external validation using a test set to rigorously assess their robustness. For test-set validation, two-thirds of the data (two replicates) were used for training, while the remaining one-third (one replicate) served as the test set. This process was repeated three times to ensure that every sample was included in both training and validation phases. In addition to spectral pretreatments, the number of latent variables (components) used in each PLSR model was fine-tuned to capture sufficient variance without overfitting. Component selection was guided by error plots, performance metrics, and analysis of regression vectors.

All models were evaluated based on key performance indicators, including the root mean square error of calibration (RMSEC), cross-validation (RMSECV), and test-set prediction (RMSEP), as well as the coefficients of determination for calibration (R^2C), cross-validation (R^2CV), and prediction (R^2P). The concentration ranges of the materials were not specifically chosen to evaluate detection limits, as the primary focus was on modeling realistic industrial fortification and adulteration scenarios. As a result, the minimum concentrations used were generally higher than the anticipated detection limits of the instruments. All data analysis and visualization were performed using R (version 4.3.0, 2023, The R Foundation for Statistical

Computing, Vienna, Austria) with the aquap2 package (Lukacs et al., 2024; Kovacs & Pollner, 2016).

4. RESULTS AND DISCUSSION

Presenting the established chemical profiles with Antioxidant Activity, Total Polyphenol Content and HPLC analysis, this following section later delves into the exploratory evaluation of spectral data based on raw spectra and PCA, then building on these previous insights introduces constructed predictive models using PLSR to predict extract concentrations and chemical parameters.

4.1. Chemical Measurement Results of the Raw Extracts

4.1.1 Antioxidant Activity and Total Polyphenol Content assay results

Figure 7 shows the Total Polyphenol Content (TPC) and Antioxidant Activity (AA) results of the raw GSE, PSE, PBE and GTE.

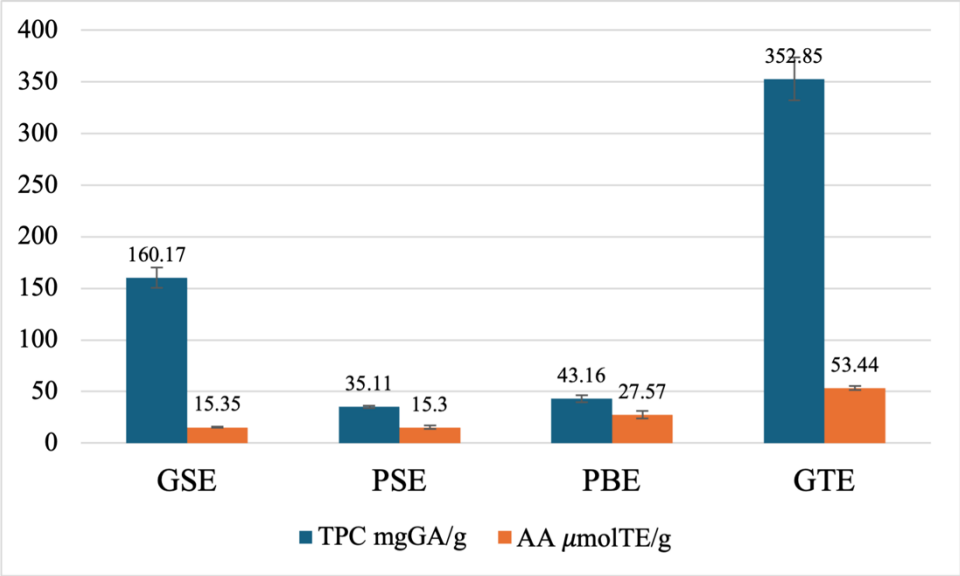


Figure 7: TPC and AA results of raw extracts

As reported in **Figure 7**, GTE showed a substantially higher TPC (352.85 ± 20.65 mg GA/g) compared to all the other extracts. GSE (160.17 ± 9.74) mg GA/g is second, while PBE and PSE have much lower and similar TPC levels (43.16 ± 3.31 and 35.11 ± 1.26). Comparatively, when it comes to the AA results GTE has the highest mean AA (53.44 ± 1.90 µmol TE/g) followed by PBE (27.57 ± 3.52 µmol TE/g). Meanwhile in case of AA, GSE and PSE have the lowest and nearly identical concentration values (15.35 ± 0.39 and 15.30 ± 1.69 µmol TE/ g).

Shapiro - Wilk Test results for normality indicated that residuals of the collected data are normally distributed for both TPC (p= 0.378) and AA (p= 0.863) with a p value greater than

0.05. The homogeneity of variances was equal across the groups according to Levene's Test with p values well above 0.05 both for TPC (p= 0.291) and AA (p= 0.670). Skewness results (TPC= 0.73 and AA=0.79) showed a perfectly acceptable asymmetry of data while kurtosis (TPC=1.94, AA= 2.02) indicated a slightly flatter distribution. Each latter statistical test supports the conclusion that the collected data does not significantly deviate from normality.

The ANOVA test resulted in extremely small p-values both for TPC and AA ($p= 2.05 * 10^{-9}$ and $p= 6.92* 10^{-7}$) which confirms that there are highly significant differences in the mean TPC and AA among the four extracts. Tukey's Post-Hoc Test in case of TPC confirmed that GTE contains significantly higher amounts than all other extracts, GSE significantly higher than both PSE and PBE, and there is no significant difference between PSE and PBE. Meanwhile Tukey's Post-Hoc Test in case of AA resulted in GTE possessing a significantly higher value than other extracts, PBE significantly higher than GSE and PSE, and no significant difference was detected between GSE and PSE.

Overall Pearson Correlation showed a positive linear relationship between the TPC and AA values with a correlation coefficient (r) of 0.81 and p-value of 0.00142, however analyzing the relationship within each different extract represented GTE with the only significant positive connection of its own (r=0.997, p-value=0.0474), almost entirely driving the strong overall positive correlation. GSE exhibited a very weak nonsignificant positive correlation (r= 0.192, p=0.877), PBE a strong nonsignificant negative one (r= - 0.970, p=0.157) and PSE a weak nonsignificant negative one (r= - 0.137, p=0.913).

Substantially, based on these results connected with previously published literature data it can be assumed that the GTE's monomeric catechin, especially epigallocatechin (EGC) and epigallocatechin gallate (EGCG) can drive both TPC and AA assays due to their high reactivity (Kupina et al., 2019). The lack of correlation between other extracts demonstrates that high TPC does not always equate to high AA, due to the type, structure and bioactivity of the contained polyphenols. In previously published literature results of TPC and AA assays are highly diverse when it comes to GSE, PSE, PBE and GTE depending on the extraction method, conditions, the source and plant variety. Generally, the AA of extracts more times than not tends to be one or two orders of magnitude higher, while TPC values are a bit lower or similar compared to the current work (Li et al.,2008; Xia et al., 2010; Ross et al., 2011; Krasteva et al., 2023; Nisca et al., 2021; Cádiz-Gurrea et al., 2014; Luo et al. 2020; Molan et al., 2009; Lewis et al., 2013; Bodoira et al., 2017). One of the similar results is the detected TPC (43.07± 3.31 mg GA/g) of Pinus sylvestris variant of PBE obtained by ultrasound assisted extraction and AA (26.66% DPPH inhibition % - compared in this work 29.35%) of Pinus nigra variant of PBE

obtained by microwave assisted extraction, reported by Nisca et al. (2021). Concerning GSE, Krasteva et al. (2023) found 111.22 ± 1.28 mg GA/g TPC in Pinot Noir GSE prepared with aqueous ethanol solution. Luo et al. (2020) measured 243 ± 7 mg GA/g TPC for GTE obtained with a combination of ultrasound-assisted extraction and deep eutectic solvents, which is one of the highest results found in literature data. Bodoira et al. (2017) experimented with different ethanol co-solvent concentrations, temperatures and solvent flows when measuring TPC of PSE and on 140 ° C with 5 g/min flow recorded the values 48.67 ± 4 mg GA/g with 95% ethanol concentration and 29.08 ± 2.29 mg GA/g with 0% ethanol concentration.

4.1.2 HPLC Measurement Results for Polyphenols

Figure 8 presents the quantified polyphenolic compounds in each raw extract after HPLC measurement.

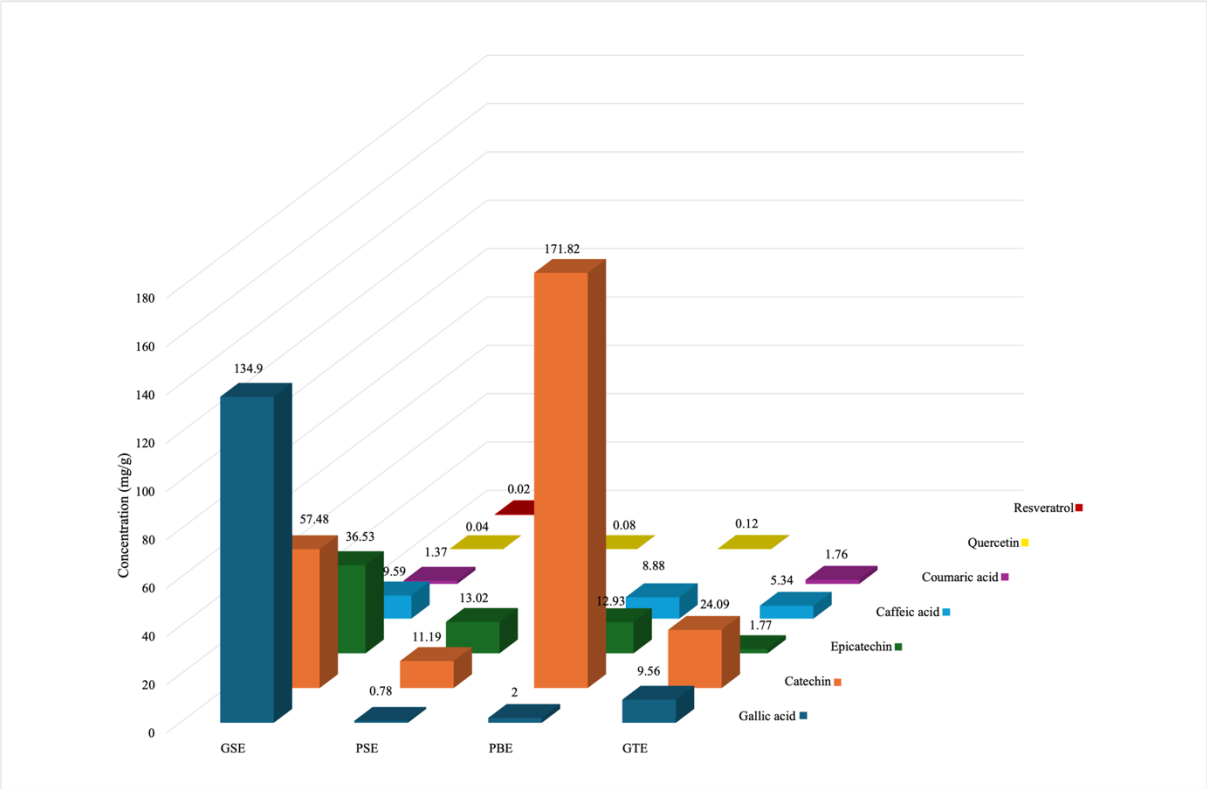


Figure 8: Quantified polyphenolic compounds in the raw extracts

In addition to the quantified compounds presented in the **Figure 8**, further identified phenolic compounds were Procyanidin C1 and C5 and Proanthocyanidin in case of all raw extracts. Luteolin was detected solely in PSE and Procyanidin B2 and B4 only in GSE. Moreover, at ~33.5 and 34.6 minutes retention times two peaks were not definable as compounds based on comparison with available standards but were present in all four extracts. This late elution time can be expected from less polar compounds with higher molecular

weights and more hydrophobic substituents, like catechin polymers, less common stilbenes, higher molecular weight proanthocyanidins.

As reported in **Figure 8**, GSE has the highest concentration of gallic acid and it also contains significant amounts of catechin and epicatechin, furthermore it contained the most identifiable and quantifiable compounds, being the only one containing resveratrol. PBE is overwhelmingly dominated by catechin, with a concentration that is the highest out of all values. GTE's profile was characterized by moderate levels of catechin and a lower, but second highest amount of gallic acid. PSE showed the lowest overall concentrations, its most abundant compounds being epicatechin and catechin. The detected high catechin content of PBE is not expected based on literature compared to GTE and GSE, however this value can differ greatly amongst different Pinus species (Villani et al.,2015; Yesil-Celiktas et al.,2009). The same can be stated about the high gallic acid content of GSE and low gallic acid content of PBE (Krasteva et al., 2023; Shi et al., 2003).

Overall exploratory Pearson correlation with TPC and AA results didn't show positive r values with almost all the concentrations measured by HPLC, which supports the idea that the compounds responsible for higher TPC and AA values were not among those quantified. Both TPC and AA values show a strong negative correlation with epicatechin ($r = -0.761$ and -0.740) and a moderate positive one with coumaric acid ($r = 0.506$ and 0.559), however both trends need further assessments and data to be called significant.

4.2. Exploratory Data Evaluation of the NIR Spectroscopy Results Based on Raw Spectra and PCA

The full range raw spectra of the measured samples are visualized on **Figure 9**., where a noticeable baseline shift can be observed at 1100 nm, due to NIRS XDS operating with two detectors. Therefore, for further analysis the 1100–2250 nm range was selected, possibly being the most informative, also excluding the upper, noisier part of the spectra between 2250- 2500 nm. The raw spectra of the measured pure extracts are visualized on **Figure 10**., while the raw spectra of pure GSE and all adultered mixtures on **Figure 11**. Sawitzky-Golay filter and MSC was chosen as pretreatments to minimize baseline shifts and slope variations due to spectral noise and other unwanted variance.

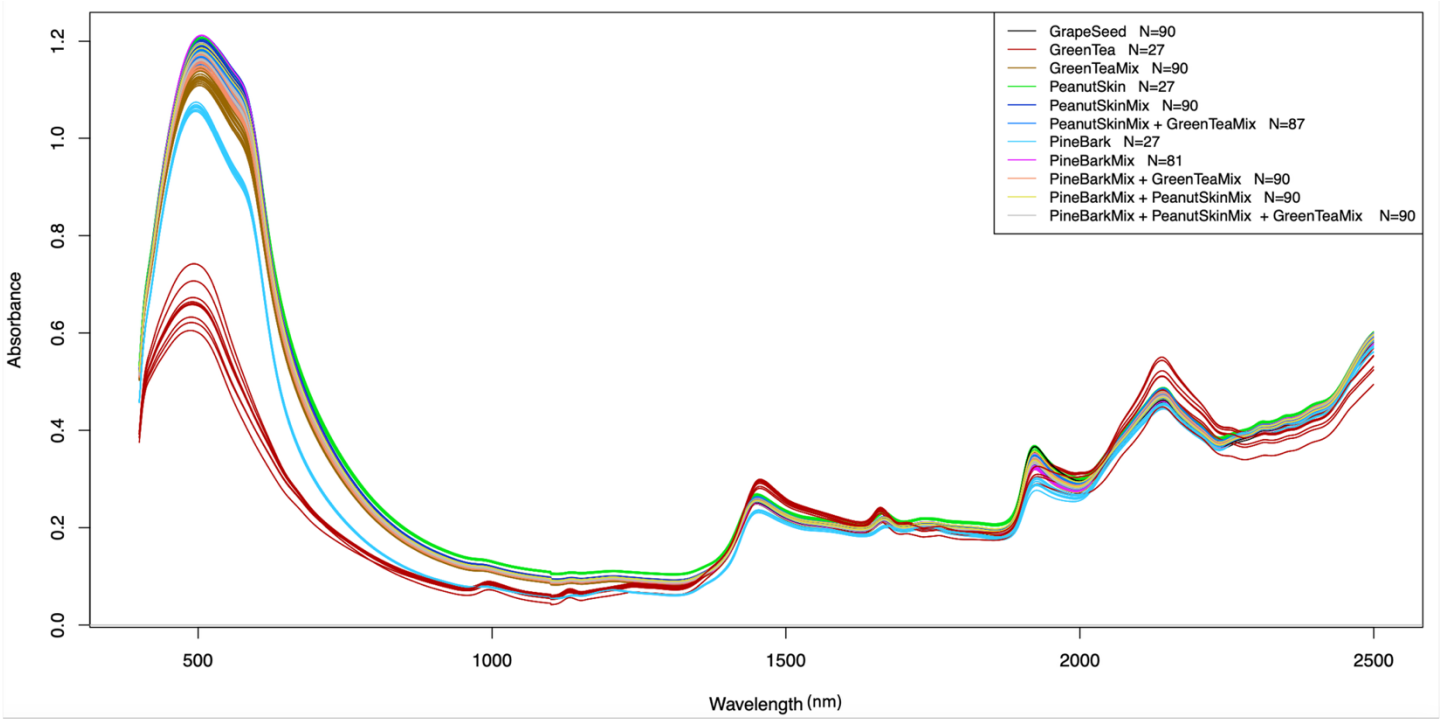


Figure 9: Full range raw spectra of samples colored by the extract type

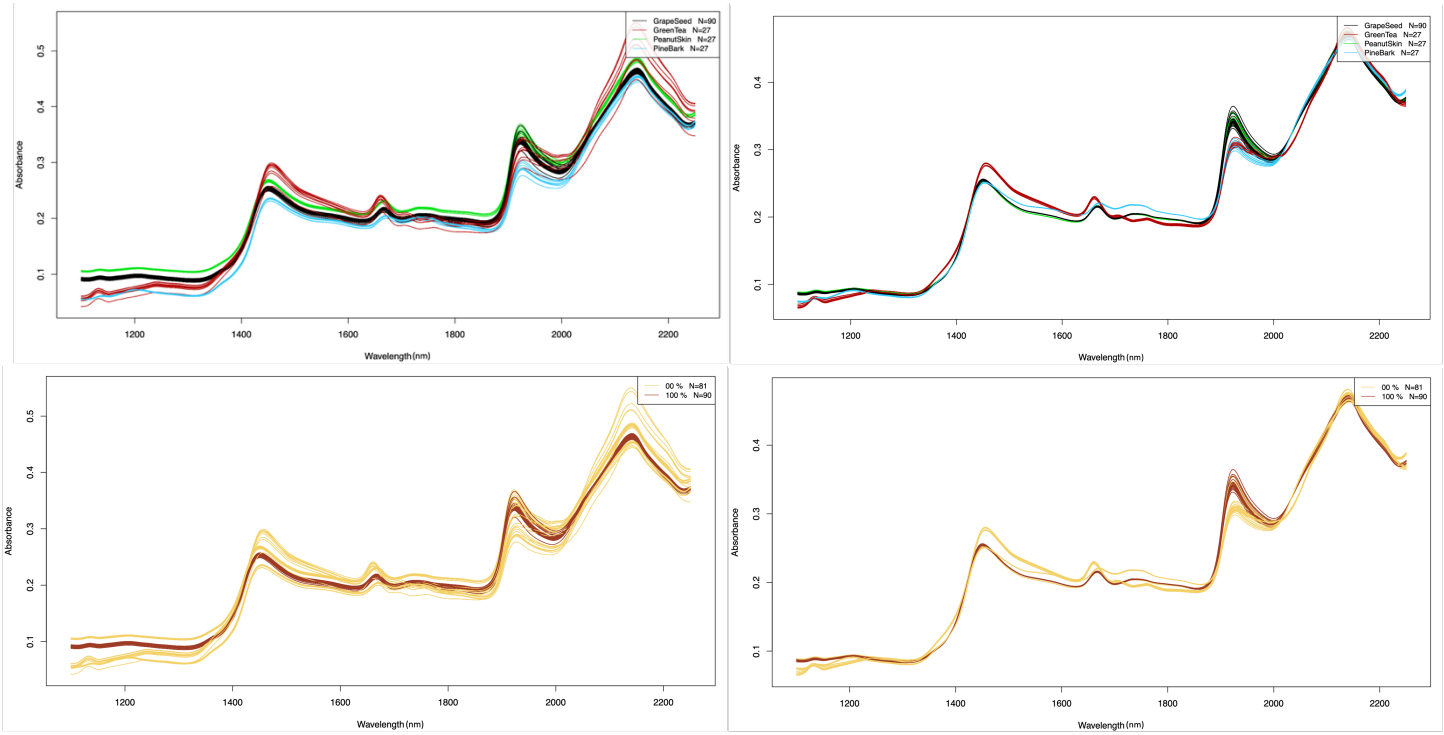


Figure 10: Raw spectra of the pure extracts colored by the extract type (above), and the concentration of GSE (below). The right side presents the raw spectra after Savitzky-Golay filter and MSC as pretreatments

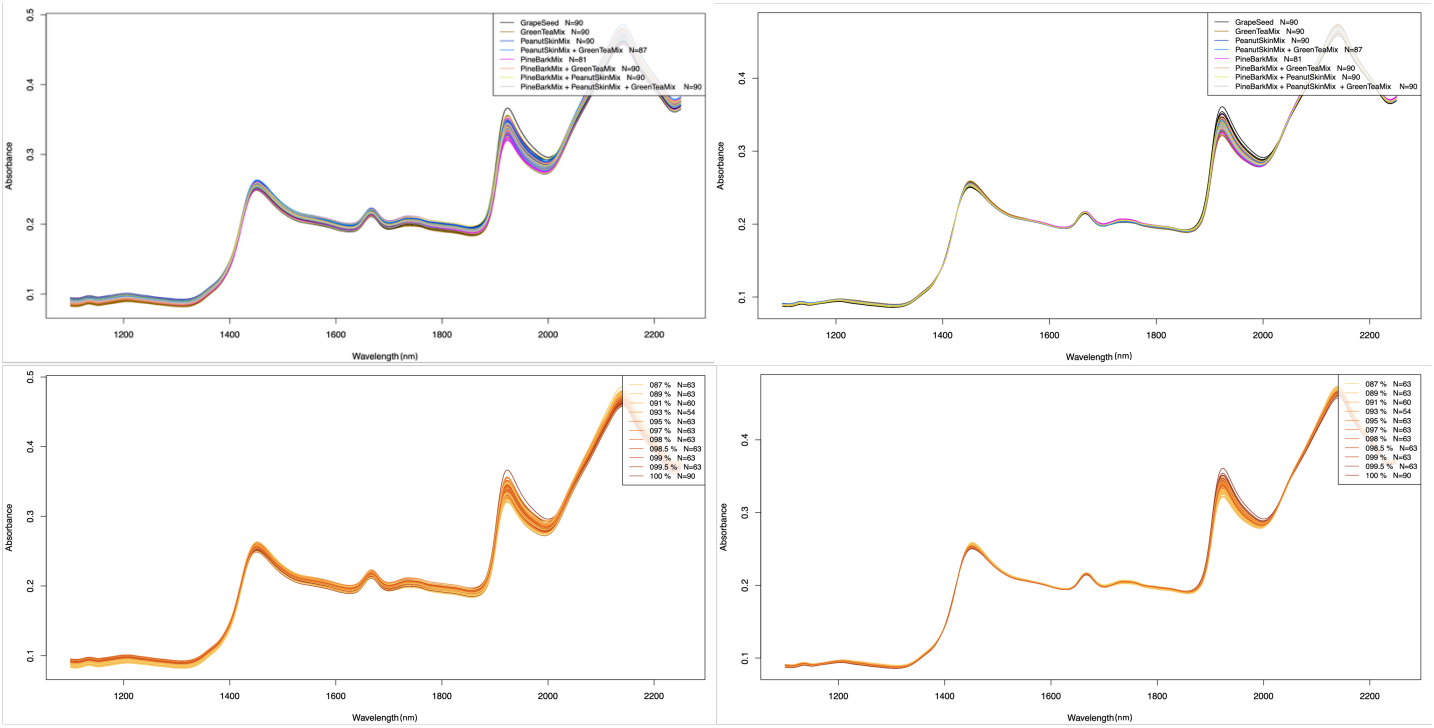


Figure 11: Raw spectra of pure GSE and all adultered samples colored by the extract and mixture type (above), and the concentration of GSE (below). The right side presents the raw spectra after Savitzky-Golay filter and MSC as pretreatments

When inspecting the raw spectra, only subtle differences can be observed between the spectra of the different samples, mainly in the 1650-1750 nm range, in the first overtone region, where C-H stretching bands can be found with the additional possibility of S-H bands. Polyphenolic compounds, like proanthocyanidins are known for containing aromatic rings where C-H bonds are present, therefore this region could explain compositional differences. There are apparent peaks at 1450 nm (second overtone region), 1950 nm (first overtone region) and 2150 nm (combination band region). Around 1400-1450 nm water molecules are known to have a strong absorption, and aromatic O-H stretching bands are also characteristic, which can be also attributed to phenolic compounds. The peak at 1950-1960 nm is associated with C=O, N-H and O-H bands. Meanwhile C-H aromatic (aryl) combination bands are typical at 2150-2160 nm (Workman Jr. & Weyer, 2012).

Following the initial application of spectral pretreatments and the selection of the prominent regions, the PCA were summarized on **Figure 12** in case of pure extracts and **Figure 13** for pure GSE and adulterated mixtures. The separation of measurement points can mainly be seen along the first and second principal component (PC 1, PC 2) in both cases.

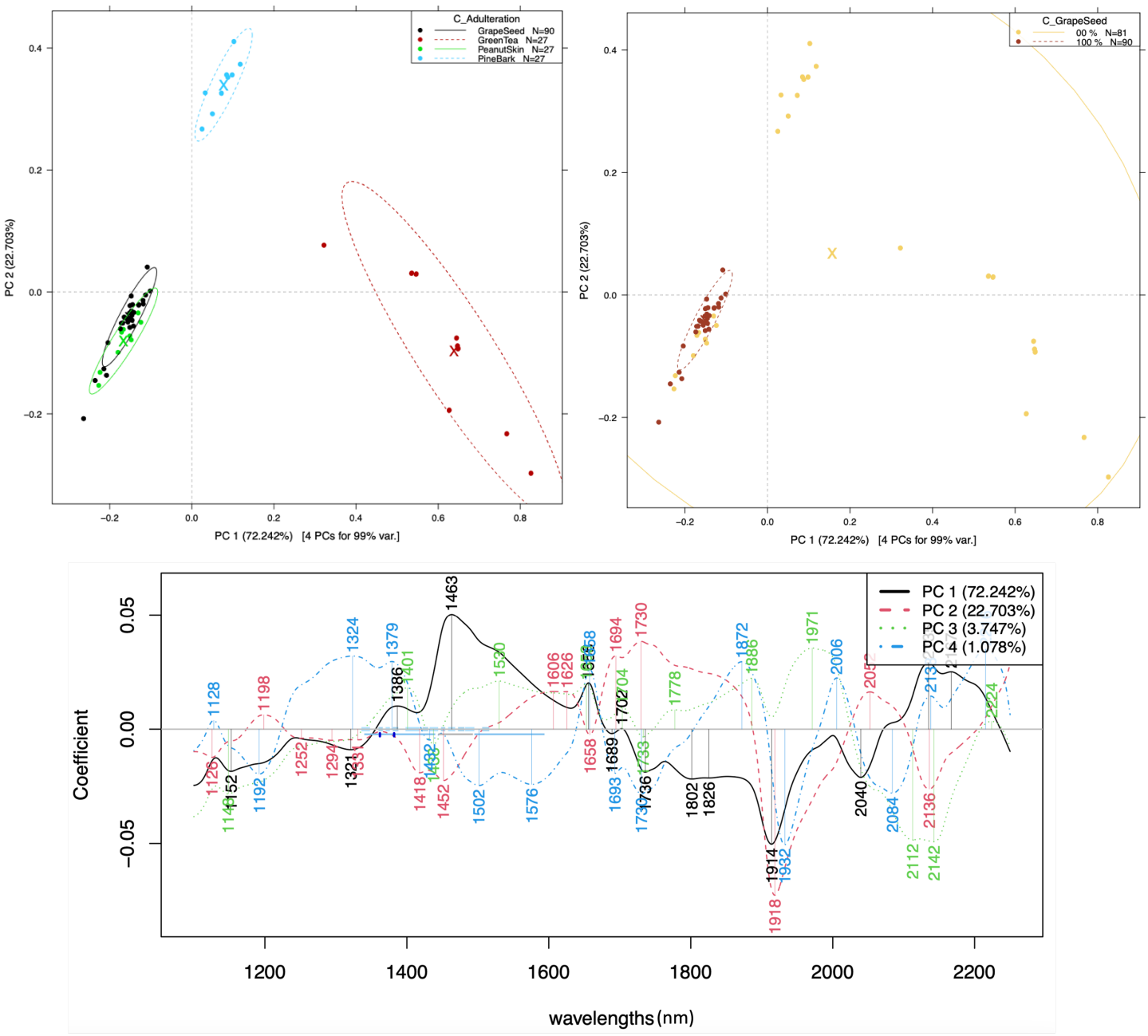


Figure 12: PCA results to separate measurement points of pure extracts, based on extract type (left score plot) and GSE concentration (right score plot), and the corresponding loading vectors after Savitzky-Golay filter and detrending as pretreatment

After observing the score plots of **Figure 12**, it is visible, that GTE separates from GSE more so along PC 1, accounting for 72.242 % of the total variance, than the other two extracts, PSE being the least distinguishable. The corresponding loading vectors demonstrate that the most important wavelength regions in this separation are mainly at 1463, 1656, 1914, 2040, 2135 and 2167 nm. The characteristic bands around the peaks at 1463 and 1656 nm are previously discussed in the second paragraph of this chapter, and they can be connected to

phenolic compounds. The 1914 nm peak in the first overtone region can be associated with O-H bands of water and C=O, which is potentially found in the functional groups of polyphenols. The mentioned peaks above 2000 nm can be identified as C-H, O-H and N-H combination bands and can indicate the presence of organic compounds like carbohydrates, hydrocarbons and proteins. The primarily wavelength regions contributing to the separation along PC 2 holding 22.703 % of the total variance are at 1452, 1730, 1918, 2052 and 2136 nm, the typical molecular assignments of which are all previously discussed (Workman Jr. & Weyer, 2012).

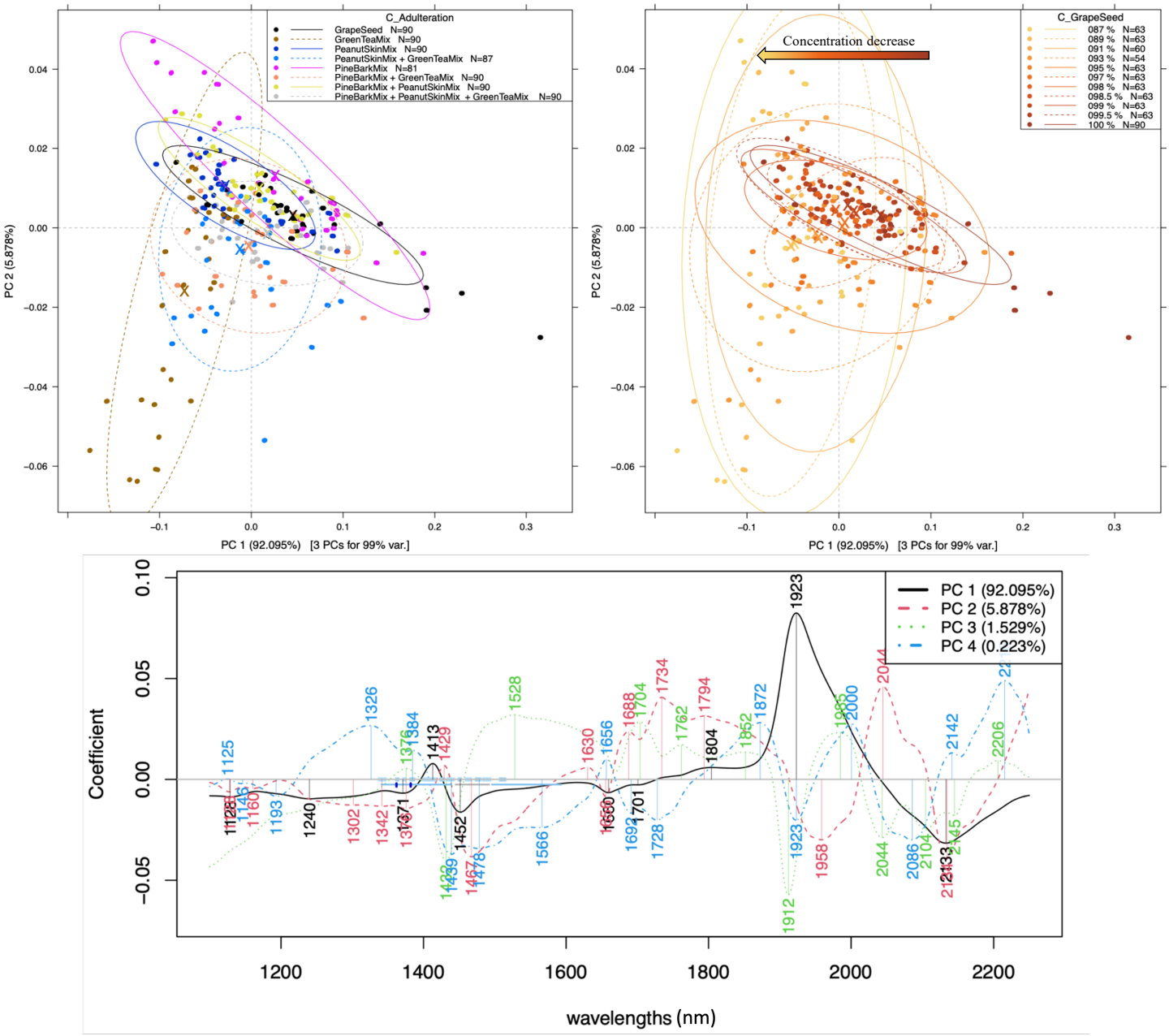


Figure 13: PCA results to separate measurement points of pure GSE and adulterated mixtures, based on extract and mixture type (left score plot) and GSE concentration (right score plot), and the corresponding loading vectors after Savitzky-Golay filter and MSC as pretreatment

Interpreting the right-side score plot on **Figure 13**, the concentration decrease of GSE can be perceived mainly along PC 1, explaining 92.095% of the total variance. With higher concentration of GSE, the score along PC1 shows an increase. By examining the corresponding loading vectors, the most significant peaks for this separation were at 1413, 1452, 1923 and 2133 nm. The peak at 1923 nm is a major positive one compared to the other ones, which indicates that higher GSE concentration is correlated with this higher absorbance, as previously mentioned strongly associated with O-H bands of water and carboxylic acid groups (Workman Jr. & Weyer, 2012). The partition of measurement points can be also observed along PC 2 adjusting for 5.878% of total variance. The left-side score plot reveals that mixtures containing PBE and GTE as additives separated most alongside this component. Prominent peaks connected were at 1467, 1734, 1958, 2044 and 2134 nm.

Overall, the separation explored with PCA, especially the linear trend observed with respect to concentration of GSE in mixtures suggests, that a quantitative model, like PLSR could be developed to accurately predict extract concentrations based on the NIR spectra of samples.

4.3. Predicting Extract Concentrations and Chemical Parameters Using PLSR

The results of predicting different extract concentrations with PLSR are presented on **Figure 14** and **15**. Comparing the models, test-set predictions for PBE and GTE concentration were exceptionally strong with a high R^2P value of 0.9927 and 0.9886 and low RMSEP of 0.2199% and 0.2814%. The low number (4 comps) of latent variables used for the GTE model suggests that GTE has a very distinct, easy to model spectral signature compared to the other extracts, which aligns with the previous PCA analysis and literature data about its chemical profile. Prediction models for GSE and PSE also presented good performance, with R^2P values above 0.93. RMSEC and RMSEP values were close to each other in case of all models, which implies reliability without overfitting. Moreover, the corresponding regression vectors show many identical observations to the PCA loadings, for example in case of the GSE model there is a positive coefficient around 1910-1930 nm, while in case of all other extracts negative peaks can be observed in this region. This matches with the PCA loadings where measurement points belonging to higher GSE concentrations were separated mainly based on this region. These agreements imply robust model fits, likely capturing the chemical differences between samples.

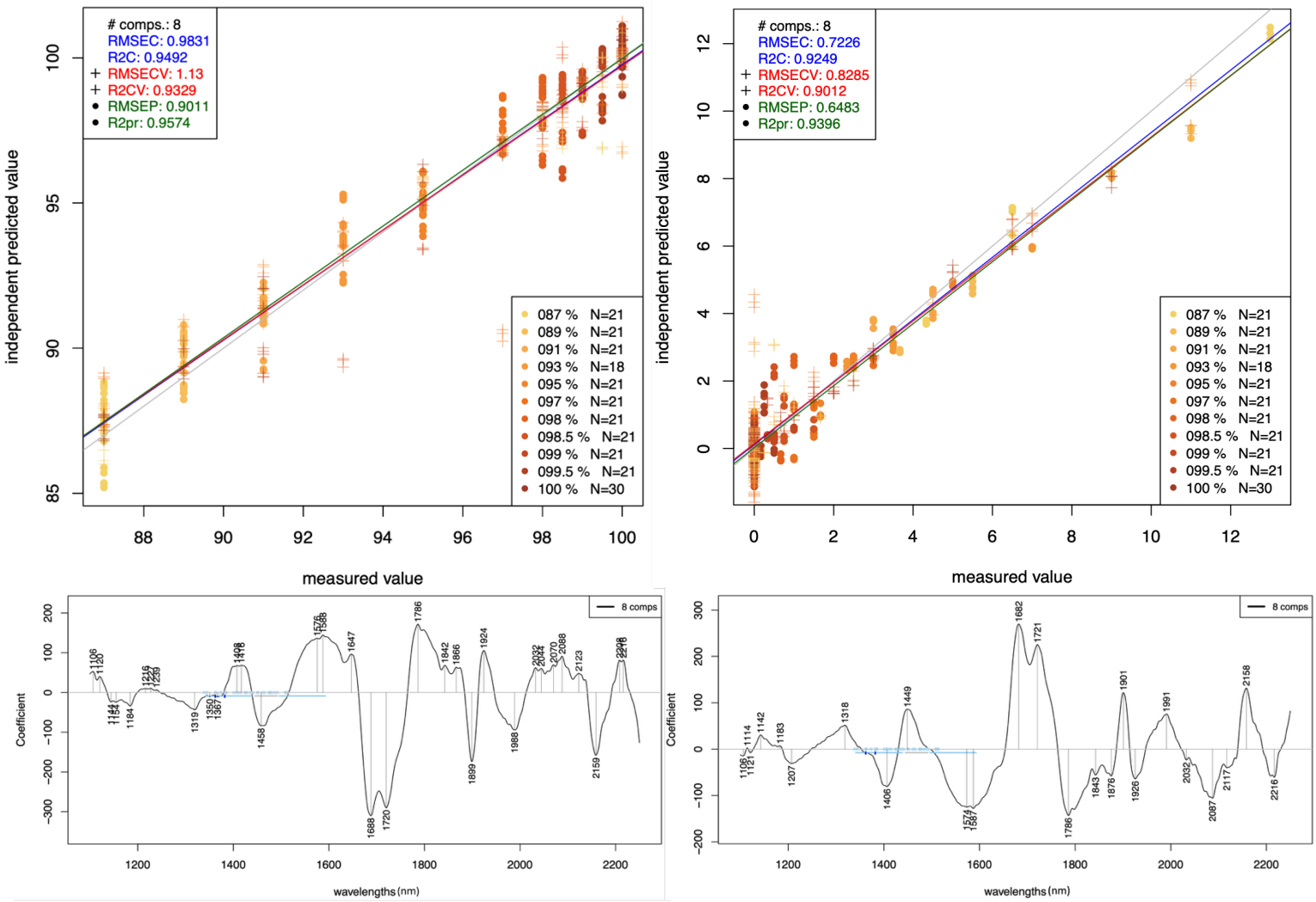


Figure 14: Test set prediction results to predict GSE (left) and PSE (right) concentration (w/w %), presented by Y-fit plots using PLSR and the corresponding regression vectors with Savitzky-Golay filter and MSC as pretreatment

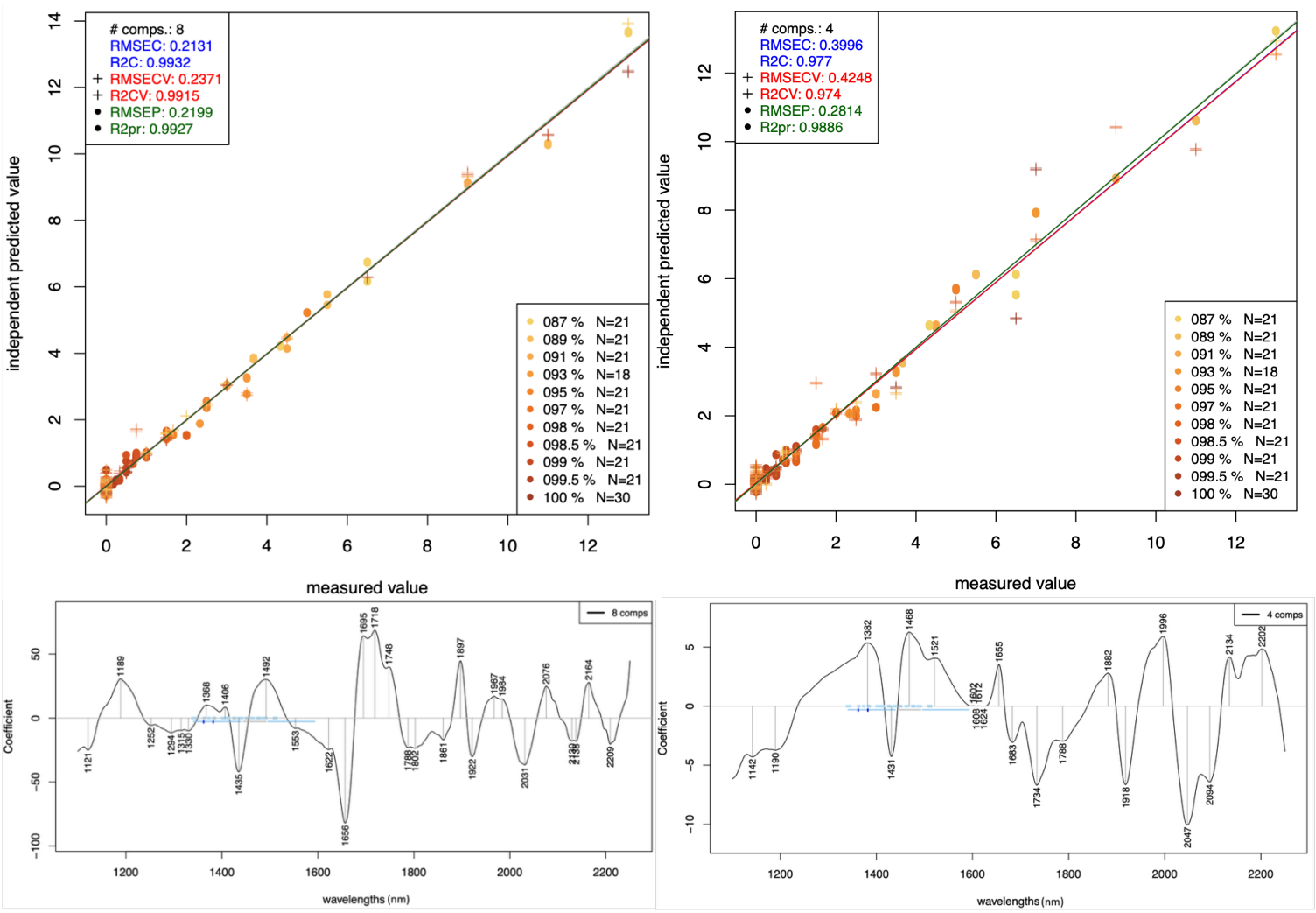


Figure 15: Test set prediction results to predict PBE (left) and GTE (right) concentration (w/w %), presented by Y-fit plots using PLSR and the corresponding regression vectors with Savitzky-Golay filter and MSC as pretreatment

Figure 16 demonstrates test-set predictions for TPC and AA values. Between these two, the model for AA shows an exceptionally high R^2P value of 0.9928 and very low RMSEP of 0.03 µmol TE/g , with very slight difference compared to the R^2C and RMSEC values. This shows that the NIR spectrum captures the molecular information directly related to the sample's functional capacity to scavenge radicals. The TPC prediction resulted in a high R^2P value of 0.9502, however also a relatively higher RMSEP of 1.9759 mg GA/g, this indicates a slightly weaker correlation between spectral data and the TPC of the samples.

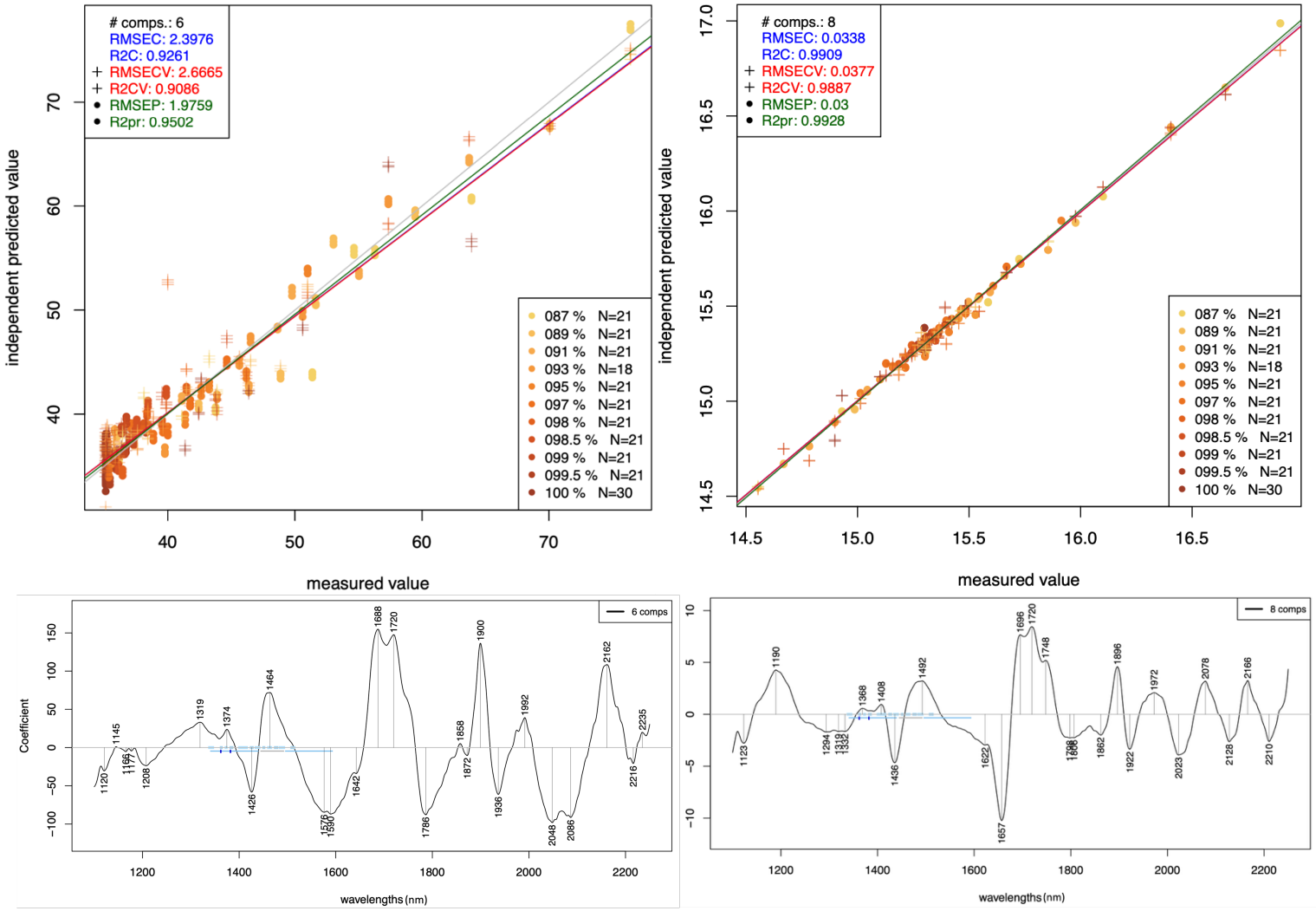


Figure 16: Test set prediction results to predict TPC (left, mg GA/g,) and AA (right, µmol TE/g) presented by Y-fit plots using PLSR and the corresponding regression vectors with Savitzky-Golay filter and MSC as pretreatment

Figure 17 and **18** displays the predictive results for individual phenolics (gallic acid, catechin and epicatechin). Amongst these the model for epicatechin is the most accurate with a tight fit to the regression line, RMSEP of 0.2304 mg/g and R^2P of 0.9642, which values have minimal difference compared to the RMSEC and R^2C. In addition, gallic acid and catechin models also show high predictive power with R^2P above 0.95. By looking at the Y-fit plots, in case of gallic acid the scattering of data points shows that the model's prediction error is elevated for higher concentrations while the model for catechin displays the greatest accuracy in the middle of the range.

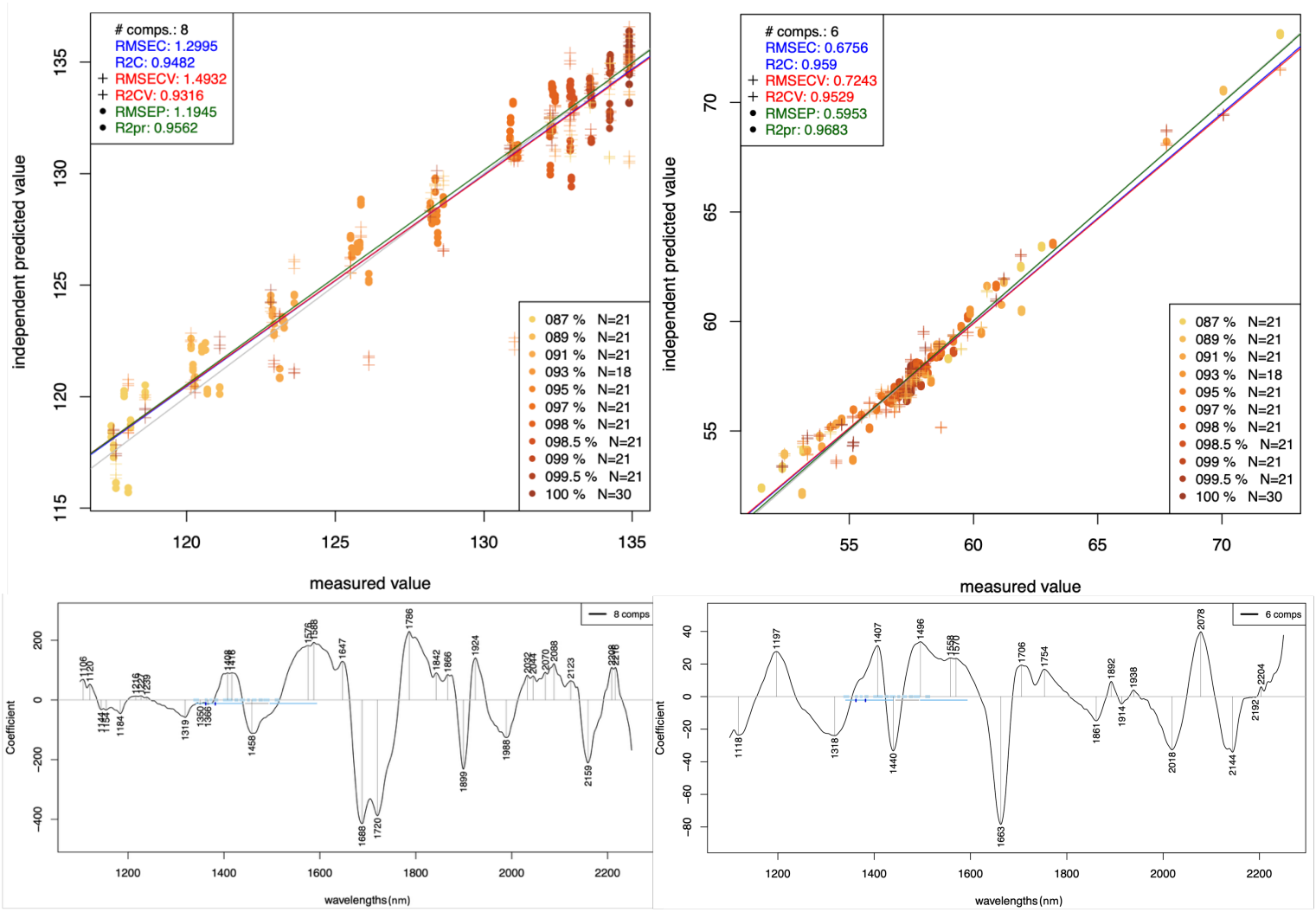


Figure 17: Test set prediction results to predict gallic acid (left) and catechin (right) content (mg/g) presented by Y-fit plots using PLSR and the corresponding regression vectors with Savitzky-Golay filter and MSC as pretreatment

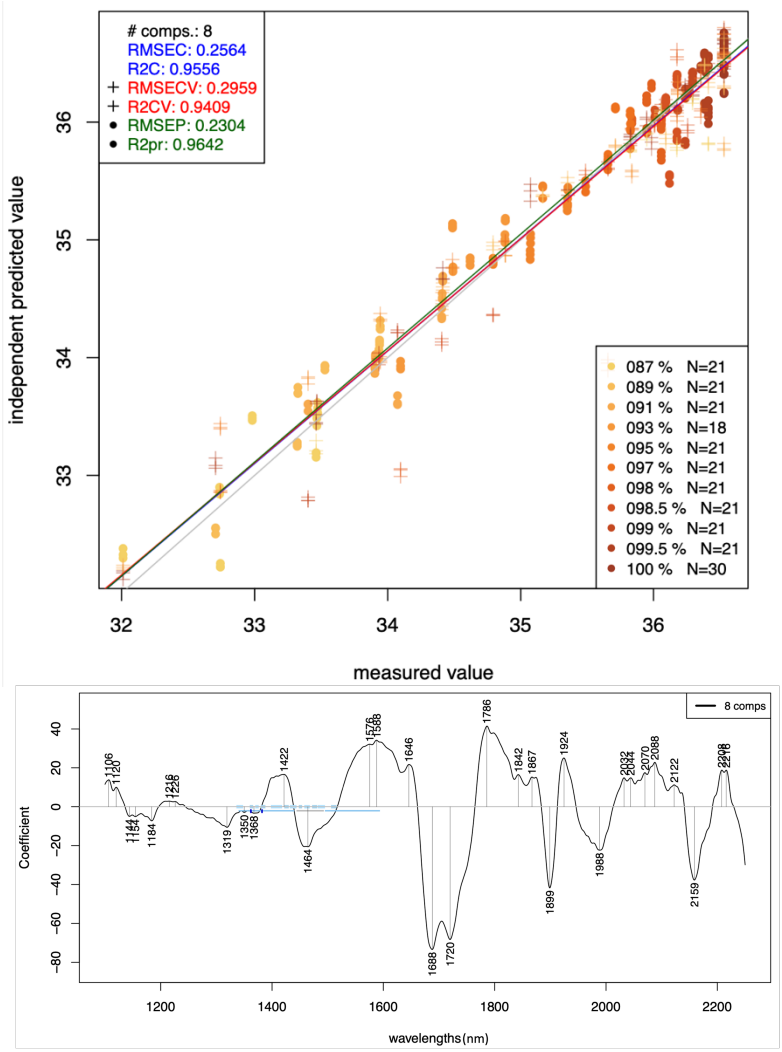


Figure 18: Test set prediction results to predict epicatechin content (mg/g) presented by Y-fit plots using PLSR and the corresponding regression vectors with Savitzky-Golay filter and MSC as pretreatment

While assessing the predictive models together, it stands out that the wavelength region between 1680-1730 nm is consistently present as significant peaks in regression vectors of different extract concentrations and chemical parameters. Subtle differences were also noticeable in this region when interpreting the raw spectra of pure extracts, moreover it also aligns with the peaks of PCA loadings. Therefore, the presupposition, that C-H stretching bands and S-H bands found in the first overtone region can have a role in explaining compositional differences connected to proanthocyanidins seems to be correct. The regression vectors present positive coefficients in this region for PSE, PBE, TPC, AA and Catechin therefore increasing absorbance at this wavelength is correlated to the increase of the predicted value in these models. On the other hand, negative coefficients can be seen for GSE, GTA, Gallic acid and

Epicatechin. PLSR models however cannot be interpreted in isolation, their behavior is defined by a complex of direct and inverse correlations between all components in the sample matrix. Overall, it can be stated that the models show robust and reliable model fits, feasible to reflect the underlying chemical differences between samples.

The scientific literature available for direct comparison of the present results is very scarce, however different matrices, containing phenolic compounds have been analyzed using NIRS combined with chemometrics, including PLSR. Zareef et al. (2019) used a portable NIR device coupled with chemometrics to predict the concentrations of different phenolic compounds, including gallic acid and L-epicatechin in congou black tea. Prediction results of classical PLS for gallic acid were R^2p= 0.8558 and RMSEP = 5.46 mg/100 g and for L-epicatechin R^2p = 0.9449 and RMSEP = 27.7 mg/100 g. In the present thesis, gallic acid, the most abundant compound in GSE according to the HPLC results, was predicted with better metrics, while the epicatechin model showed similar results. Alongside the fact, that the measurements were taken with a benchtop device, compared to a portable one, the differences can probably be also attributed to the more complex matrices used in this work, highlighting the matrix dependency of the technique. Sun et al. (2020) assessed the quality of instant green tea applying quantitative PLS models for the measured spectra of a benchtop FT-NIR spectrometer and obtained the modeling results R^2p = 0.958 and RMSEP = 1.220 % for catechin and R^2p = 0.966 RMSEP = 0.159 % for epicatechin, which values are in line with the current results. Zareef et al. (2021) coupled benchtop NIR spectroscopy with multivariate analysis for the classification and prediction of antioxidant properties of walnut and obtained a PLS model for TPC with R^2p = 0.9361 and RMSEP = 0.205 GA/100 g. These results, when compared to this thesis show a slightly better correlation and can once again prove matrix reliance.

5. CONCLUSIONS AND SUGGESTIONS

This section summarizes the key findings of the thesis, reflecting on the implications of the results and the potential of NIR spectroscopy as a rapid and non-destructive method for quality control in plant extracts, specifically GSE.

The initial chemical characterization using conventional methods (TPC, AA and HPLC assays) established distinct and significant differences between authentic GSE and its adulterants PBE, PSE and GTE. However, the limited correlation between TPC, AA and individual polyphenolic concentrations highlighted the difficulty of relying solely on targeted assays for extract authentication, given structural diversity and overlapping bioactivity of polyphenolic compounds. Moreover, previous studies indicate that the outcomes of these assays can vary significantly depending on the extraction method, source and plant variety.

Exploratory analysis of the NIR spectral data using PCA after applying pretreatments effectively distinguished the pure extracts from one another and revealed a concentration dependent pattern for GSE in the adultered mixtures. This demonstrated that NIR Spectroscopy is highly sensitive to compositional variations between these chemically similar plant-based materials.

Building on the previous analysis PLSR models were developed and validated to quantify both the concentration of each extract and their chemical properties. Regarding extract quantification the concentrations of GSE, PBE, PSE and GTE were predicted with high accuracy, achieving R^2P greater than 0.93 and low RMSEP. The models for PBE and GTE were particularly robust ($R^2P > 0.98$), confirming their distinct spectral signatures. The models also proved highly effective at predicting chemical parameters directly from the spectra. The prediction for AA was exceptionally strong ($R^2P> 0.99$, RMSEP=0.03 μmol TE/g) and overall reliable models were established for TPC and individual phenolic compounds.

In summary, it can be stated that the specified goals of the thesis were achieved and the assessments confirmed that NIR spectroscopy can be a powerful, reliable and efficient tool in the quality control of GSE. When coupled with appropriate chemometric strategies it offers a viable alternative to time-consuming and destructive wet chemical methods, providing means of rapid authentication and quality assessment of chemically similar polyphenol rich extracts.

The results of this thesis suggest that:

- In the future more polyphenolic extracts and different variants of the current ones could be investigated.

- Moreover, to expand for field applications, the models could be tested on portable and handheld devices.
- Lastly, for routine industrial quality control, classification based chemometric methods like SIMCA (Soft Independent Modeling of Class Analogy) or PLS-Discriminant analysis could also be explored, to build a robust authentication system that functions like an alarm.

6. SUMMARY

For a condensed overview of the entire thesis, the main insights are distilled in the summary that follows.

Grape seed extract (GSE) as a plant-based dietary supplement is a highly valued commodity on the market, due not only to its well-proven health benefits, but also its contribution to waste management and circular economy as a product of agri-food industry byproduct valorization. The absence of rapid, cost-effective and sufficiently selective quality control methods capable of characterizing it on the market lead to misclassification, batch to batch variability and economically motivated adulteration.

This thesis aimed to assess the performance of Near-Infrared Spectroscopy (NIRS) combined with chemometric modeling as a non-destructive, rapid and green analytical alternative for quantifying polyphenols and detecting adulterants in GSE.

Authentic samples of pure GSE, and other proanthocyanidin rich, extracts such as pine bark (PBE), peanut skin (PSE) and green tea (GTE) were prepared along with binary, dual and ternary adulterated mixtures at ten concentration levels, in line with the industrial practice of adulteration. The pure samples were analyzed using Antioxidant Activity (AA, DPPH), Total Polyphenol Content (TPC, Folin-Ciocalteu) assays and High-Performance Liquid Chromatography (HPLC) for specific phenolic profiling. All samples underwent NIR spectral acquisition with a benchtop NIR XDS instrument, recording spectra within the 400–2500 nm wavelength range. The collected spectral data was subjected to wavelength range selection (1100–2250 nm), pre-treatments and multivariate analysis including Principal Component Analysis (PCA) and Partial Least Squares Regression (PLSR).

The initial chemical characterization revealed distinct and complex chemical profiles between GSE and its adulterants, where GTE showed exceptionally high TPC and AA while PBE was uniquely rich in catechin. The results of univariate statistical comparison highlighted the structural diversity and possible overlapping bioactivity of polyphenolic compounds.

PCA results of NIRS effectively visualized group separations and spectral variance associated with GSE concentration. PLSR models achieved strong prediction performance for extract concentrations, TPC, AA and individual phenolic compounds (namely gallic acid, catechin and epicatechin), with high coefficients of determination (R^2 >0.9) and low prediction errors. The models identified very specific spectral fingerprints for PBE, GTE concentrations (R^2P > 0.98), and AA (R^2P> 0.99, RMSEP=0.03 μmol TE/g).

The findings in this thesis confirm that NIRS, supported by chemometrics, with applicable predictive models can be a viable method for polyphenol quantification and adulteration detection in GSE and other plant extracts. Its rapid, solvent free, and scalable nature makes it an attractive tool for possible future developments to improve quality control strategies in the food industry, especially the dietary supplement sector.

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The assisting, minor usage of AI is detailed in the attached AI usage declaration according to Rector's Decree No. 11/2025 (VIII. 29.), at the end of this document, after the Expression of Gratitude.

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Thank you!

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