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Comprehensive quantification of flavonoids and salicylic acid representative of Salix spp. using microLiquid Chromatography-Triple Quadrupole Mass Spectrometry: the importance of drying procedures and extraction solvent when performing classical solid-liquid extraction

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ABSTRACT

Willow (Salix spp.) is gaining an increasing interest as a fast-growing tree with high biomass yield from low agricultural inputs, which contains potentially bioactive compounds. The present work aimed to develop a high-yield extraction procedure combined with robust, sensitive and fast microLiquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-MS/MS) based method for comprehensively quantifying flavonoids and salicylic acid in the bark of *Salix* spp. We have investigated the effect of freeze- and oven-drying procedures and five extraction solvents on the yield of individual flavonoid and salicylic acid when performing classical solid-liquid extraction. The freeze-drying was the best drying procedure for preserving monomeric and polymeric flavan-3-ols, whereas other flavonoids were less affected. Salicylic acid was not affected by the drying procedures. The best extraction solvent in terms of the yield of individual flavonoid among the tested solvents in this study was the combination of methanol acidified with 1% hydrochloric acid. LC-MS/MS method has shown a high recovery percentage (≥80%), good precision and overall robustness.

1. Introduction

Phenolic compounds (PCs) are a diverse group of compounds produced by a magnitude of different plant species [1]. PCs have been used for many years as antioxidants and anti-microbial agents, pharmaceuticals, nutraceuticals and antiseptics, food supplements and feed additives, and plants are a natural source of these high-value compounds [2]. The applications of PCs are as diverse as their physicochemical properties. PCs can be classified based on their structure with the presence of one (simple phenols) or more (polyphenols) phenol groups and hydroxyl groups. The flavonoids are the largest class of polyphenols, which can be further divided into several subclasses: flavan-3-ols (monomeric and polymeric structure), flavones, flavonols, flavanones, isoflavones, anthocyanidins [2]. Another class of PCs is classified as phenolic acids, which are simple phenols sharing only one phenol group and carboxylic acid group, such as salicylic acid [3]. Willow (*Salix* spp.) has been previously reported to contain a high concentration of catechin as the main flavonoid and salicylic acid as the primary phenolic acid [4–6]. However, metabolomics studies of willow bark and leaves revealed a complex mixture of flavonoids belonging to different flavonoid subclasses, mainly flavanols, procyanidins (condensed tannins) and flavonols [7]. There is an increasing interest in willow as a bioeconomic crop due to the environmental, social, and economic benefits of growing and using plant biomass as a natural source of flavonoids and salicylic acid [6]. The differences between chemical structures and the classification of flavonoids that have been considered for this study are presented in Fig. 1.

Liquid Chromatography - Triple Quadrupole Mass Spectrometry (LC-MS/MS) is a superior technique for the characterization and determination of polyphenols and has been extensively used for the quantification of PCs in a variety of plant species [8–11]. Furthermore, the use of a microLC system has several advantages over regular LC systems such as lower consumption of solvents, smaller columns, and lower flows. Altogether improved sensitivity of analysis can be achieved due to lower ion suppression and contamination reaching the detector [12,13]. Triple Quadrupole Mass

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Flavonoids

Flavan-3-ols

Monomeric flavan -3-ols	C2	C3	3 (R_1)	5 (R_2)	7 (R_3)	3' (R_4)	4' (R_5)	5' (R_6)
(-)-Catechin	S	R	OH	OH	OH	OH	OH	H
(-)-Epicatechin	R	R	OH	OH	OH	OH	OH	H
(-)-Gallocatechin	S	R	OH	OH	OH	OH	OH	OH
(-)-Epigallocatechin	R	R	OH	OH	OH	OH	OH	OH
(-)-Catechin gallate	S	R	-O-gallate	OH	OH	OH	OH	H
(-)-Gallocatechin gallate	S	R	-O-gallate	OH	OH	OH	OH	OH

Polymeric flavan -3-ols (CT)	Monomers	Linkage
Procyanidin A2	(+)-epicatechin, (+)-epicatechin	4β-8,2β-O-7
Procyanidin B1	(-)-epicatechin, (+)-catechin	4β-8
Procyanidin B2	(-)-epicatechin, (-)-epicatechin	4β-8
Procyanidin C1	(-)-epicatechin, (-)-epicatechin, (-)-epicatechin	4β-8

Flavonols

aglycones	5 (R_1)	7 (R_2)	3' (R_3)	4' (R_4)	5' (R_5)
Quercetin	OH	OH	OH	OH	H
Kaempferol	OH	OH	H	OH	H

glycosides	3 (R_1)	8 (R_2)	3' (R_3)	4' (R_4)
Rutin	-O-Rutinose	H	OH	OH

Flavanone

	5 (R_1)	7 (R_2)	3' (R_3)	4' (R_4)
Naringenin	OH	OH	H	OH

Phenolic acids

Hydroxybenzoic acids	2 (R1)	3 (R_2)	4 (R_3)	5 (R_4)
Salicylic acid	OH	H	H	H

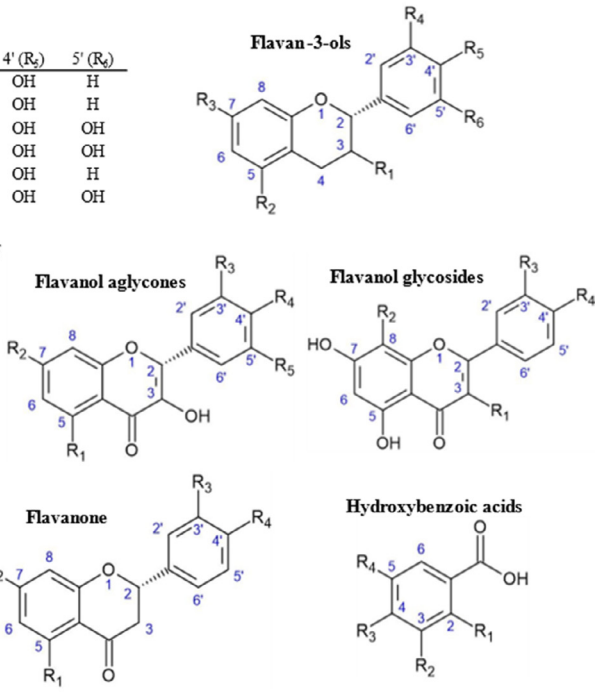

Fig. 1. Phenolic compounds (PCs) considered for this study, classification in subclasses, and differences in chemical structures.

Spectrometry provides highly selective and sensitive analyses since the quantification is based on the mass/charge (*m/z*) ratio of the precursor ion and its fragment, using unique fragmentation patterns of each compound. The most significant advantage of triple quadrupole technology is the operation of Multiple Reaction Monitoring (MRM) scan mode, which enables the quantification of many PCs simultaneously. The drawback of this technique is the prerequisite of standards for the optimization of compound-dependent parameters. Due to the progress in chemical synthesis, purification techniques, and general widespread use of LC-MS/MS, the assortment of commercially available standards has increased over the past ten years. Schoedl et al. have quantified 13 PCs in leaves of grapevine [14]. In the study of Oliva et al. (2021) high number of PCs were quantified and identified in the curry leaves, hemp and blueberry [8]. Maize flavonoids have been quantified in the study of Cocuron et al. (2019) [9]. Chromatographic separation is another critical aspect of this technique. However, complete separation of PCs is not required if compounds are not isomers and have a molecular weight difference of more than 0.5 Da. The quantification of isomers requires complete separation on a chromatographic column. The stationary phase of the column and the mobile phases used in the system are prerequisites for good binding affinity and separation. Most analytical methods for quantitative and qualitative analyses of PCs use RP-C_{18} columns, however phenyl-hexyl columns are also widely used in combination with the mobile phase composed of formic acid in water and acetonitrile [11,15–18]. The physicochemical properties of PCs such as molecular weight and lipophilicity are known to influence the retention time and ionization potential. Electro Spray Ionization (ESI) in negative ionization mode has been reported in most LC-MS methods, when analyzing PCs [8,9,11].

Developing robust sample preparation protocols is essential for preserving PCs in plant material. Further analyses can be preserved by drying, milling, and storing at −20 °C. These steps inhibit enzymatic degradation and deter microbial growth. Since air-drying is slow and metabolic processes may continue after collection, methods such as freeze-drying and oven-drying are more appropriate. Several studies have focused on the effects of drying procedures for PCs extraction and stability. Oven-drying and air-drying have been investigated with no differences between drying procedures on phenolic constituents at 30 °C. However, at higher temperatures of 70 °C, the concentration of some phenolic compounds decreased [19]. Julkunen-Tiitto et al. and Sorsa et al. showed that air drying with the addition of heat (60 °C and 90 °C) decreased the concentration of most flavonones, flavan-3-ols, and flavones, particularly catechin and total condensed tannins [20]. On the other hand, freeze-drying at −30 °C was a more effective way to preserve the PCs [20]. Further, milling and storing temperatures have been shown to impact the preservation of PCs [21,22].

Extracting PCs from plant material for quantification is a challenging task, as the physicochemical properties of PCs differ greatly between compound classes and subclasses. Solid-liquid extraction is still the most used extraction technique for plant material [23]. When performing solid-liquid extraction, analytes are extracted from the solid (plant material) to the liquid (extraction solvent) depending on the physicochemical properties of PCs [23]. Methanol, acetone, and ethanol are the primary organic solvents used when extracting PCs from dry plant material [20,24–26]. Since polymeric flavan-3-ols have a higher number of hydroxyl groups, they are more hydrophilic than other flavonoids. Therefore, the extraction of condensed tannins has been shown to be effective with hot water [19,24]. However, when using non-hot water, the yield was lower [25]. Compared with other solvents, Galgano et al. showed that deionized water yielded the lowest concentration of total phenolics, flavonoids, and condensed tannins compared to methanol and ethanol [25]. Methanol yield was higher for total phenolics and tannins but lower for total flavonoids. Furthermore, the extraction kinetics showed the highest yield of PCs for the first hour of extraction [25]. Similarly, it has been previously reported that extraction for longer than 120 min did not increase the extraction of tan-

nins [24,27]. Different models can help to calculate the polarities of different PCs and predict the extraction solvent [23]. However, these models do not consider the complexity of the plant material. Therefore, practical extraction yield experiments are essential to perform. In addition to the choice of solvent, different hydrolysis steps can be applied to the sample to either increase the yield of PCs or modify their chemical structure. Acidification has been previously reported to increase the yield during extraction, where 1% HCl had the highest yield of PCs in grape pomace compared to 0% and 0.5% HCl [28]. Acidic and alkaline hydrolyses can be used to both solubilize plant tissue to release the bound PCs from the plant cell wall matrix [24,29], as well as to disrupt the glycosidic bonds to release aglycones in the case of phenolic glycosides [6,24]. The increase in temperature up to 60 °C and 90 °C typically promotes the phenolic glycosides hydrolyses [6,24]. Enzymatic hydrolysis has also been a widely used technique to release aglycones for further quantification [29].

This work aimed to establish an efficient solid-liquid extraction combined with a robust microLC-MS/MS method to comprehensively quantify monomeric flavan-3-ols, procyanidins (condensed tannins), flavonols, flavanones, and salicylic acid representative of *Salix spp*.. Chemical structures are reported in Fig. 1. Using this robust microLC-MS/MS method, we investigated the effects of drying procedures during the sample preparation (oven-drying versus freeze-drying) and extraction solvent (use of water, methanol, and acidification) on the yield of individual flavonoids and salicylic acid. To our knowledge, detailed quantitative investigation of the extraction yields of the individual flavonoid and salicylic acid using different solvents in willow bark has not been performed before.

2. Experimental

2.1. Chemicals and standards

The following standards were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), chemical purity (CP) and CAS number listed below: catechin ≥98% (18,829–70–4), epicatechin ≥98% (490–46–0), gallocatechin ≥98% (3371–27–5), epigallocatechin ≥97% (970–74–1), catechin gallate ≥98% (130,405–40–2), gallocatechin gallate ≥98% (4233–96–9), procyanidin B1 and B2 90% (20,315–25–7 and 29,106–49–8), procyanidin A2 99% (41,743–41–3), procyanidin C1 90% (37,064–30–5), kaempferol ≥97% (520–18–3), quercetin ≥95% (117–39–5), rutin ≥94% (207,671–50–9) and salicylic acid 99% (69–72–7). Naringenin ≥96.0% (67,604–48–2) was purchased from Thermo Scientific (Waltham, MA, U.S.A). The following labelled standards were purchased: catechin-2,3,4–$^{13}C_3$ 99 atom% ^{13}C (98% CP), gallocatechin-2,3,4–$^{13}C_3$ ≥99 atom% ^{13}C (≥97% CP), catechin-2,3,4–$^{13}C_3$ gallate ≥99 atom% ^{13}C (≥97% CP), salicylic acid-D_4 certified reference material (78,646–17–0) from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and enterolactone-2,3,5–$^{13}C_3$ (918,502–72–4) from Toronto Research Chemicals (Toronto, ON, Canada). The following chemicals were purchased from Sigma-Aldrich (Merck-Millipore, Merck KGaA, Darmstadt, Germany): dimethyl sulfoxide (DMSO), formic acid (FA, LiChropur 98–100% LCMS grade), and hydrochloric acid (HCl, 37%). Methanol (MeOH, HiPerSolv Chromanorm) and acetonitrile (ACN, HiPerSolv Chromanorm) were purchased from VWR Chemicals (Radnor, PA, U.S.A.). For the enzymatic hydrolysis, β-glucuronidase type H-1 from *Helix pomatia* was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Sodium acetate was obtained from Merck (Darmstadt, Germany), and glacial acetic acid was from Fluka/Sigma-Aldrich (Thermo Fisher Scientific, Waltham, MA, U.S.A; Merck KGaA, Darmstadt, Germany). All solvents used were of HPLC grade. Ultrapure water was obtained using Millipore's Milli-Q Synergy purification system (Merck KGaA, Darmstadt, Germany).

2.2. Preparation of standards and standard curve for quantification

All non-labelled standards were dissolved in DMSO at a concentration of 1 or 5 mg/mL, except for salicylic acids, which was dissolved in methanol. Labelled standards: catechin$^{13}C_3$, gallocatechin$^{13}C_3$ and catechin$^{13}C_3$ gallate were dissolved in DMSO at 1 mg/mL, salicylic acid-D_4 was dissolved in acetonitrile in concentration 100 µg/mL, and $^{13}C_3$-enterolactone was dissolved in acetonitrile in a concentration of 1 mg/mL. Two working solutions were prepared, one containing all non-labelled standards (ST mix) and another one containing all the labelled standards (IS mix) in a working solvent of 5% ACN (v/v) and 1% FA (v/v) in water. The final concentrations of the IS mix were 100 ng/mL for (catechin-2,3,4–$13C_3$, gallocatechin-2,3,4–$13C_3$, catechin-2,3,4–$13C_3$ gallate) and 20 ng/mL for (salicylic acid-d_4 and $^{13}C_3$-enterolactone). The labelled standards were used as internal standards (IS) for quantifying PCs in bark samples. The standard curve was constructed to contain all the labelled and non-labelled compounds.

The analyte/internal standard concentration ratio was plotted against the analyte/internal standard peak area ratio as a linear regression curve with 1/x weighting, which puts emphasis on lower-value points [30]. The standard curves were automatically calculated in Analyst software 1.7.1 from AB Sciex (Framingham, MA, USA). In cases when a specific internal standard was unavailable, another internal standard was applied based on the similarities in physicochemical properties and retention time (RT). The concentrations of all the standards in the standard curve were based on the knowledge of their ionization potential in the ESI source. The standards were divided into three concentration ranges shown in Table 1. As described in the European Medicines Agency guideline on Bioanalytical Method Validation the lower limit of quantitation (LLOQ) was accepted as the lowest standard on the standard curve, and the upper limit of quantification (ULOQ) was the highest; in both cases the analyte response was within the accuracy range of 80 to 120% [31,32]. Standard curves were constructed with 12 calibration points, however, depending on the analyte responses different number of calibration points has been used, standard curves for each analyte are shown in Supporting information (Figure S4). All calibration curves showed good linearity throughout the used range of concentrations and in accordance with the European Medicines Agency guideline on Bioanalytical Method Validation (Table 1, Figure S4) [31].

2.3. microLC-MS/MS

The chromatographic separation was performed on microLC 200 series from Eksigent/AB Sciex (Redwood City, CA, USA) coupled with a QTrap 5500 mass spectrometer from AB Sciex (Framingham, MA, USA). For chromatographic separation a Kinetex 1.7 µm Phenyl-Hexyl (100 × 2.1 mm) has been used. The temperature of the column oven was 30 °C. Mobile phases consisted of solvent A (1% FA (v/v) in water) and solvent B (0.1% FA (v/v) in ACN). The gradient started at 10% of solvent B for 0.5 min., followed by an increase in solvent B for 9 min. until 90% solvent B and was kept isocratic for 0.5 min. The total chromatographic run was 10 min. The columns were equilibrated for 3 min with 10% of solvent B at the beginning of each run. The sample injection was 5 µL, and the temperature of the autosampler racks was 20 °C. The flow of the system was 60 µL/min. The flow of the method was optimized by series of injections of ST mix with varying flow 50, 55, 60, 65 and 70 µL/min and calculating the number of theoretical plates (N). The PCs were measured in MRM mode. The compound-dependent parameters were optimized manually for each compound by syringe infusion of pure standard and are shown in Table S1. The dwell time was set to 15 msec, and the Entrance Potential (EP) was at −10 eV. The ionization of compounds was performed with

Table 1
Three concentration ranges of non-labeled standards (ST mix) of the standard curve and their respective calibration coefficient, linear regression and Lower Limit of Quantification (LLOQ).

Compounds	Correlation coefficient (r)	Linear regression ("1/x" weighting)	LLOQ (ng/mL)	LLOQ accuracy (%)
		Range 1: 0.0195 – 20 ng/mL		
Naringenin	0.9998	$y = 0.126\ x + 0.000868$	0.327	105
Salicylic acid	0.9961	$y = 0.0474\ x + 0.134$	0.310	98.9
		Range 2: 0.0488 – 100 ng/mL		
Catechin	0.9992	$y = 0.0118\ x + 0.00191$	0.380	97.5
Epicatechin	0.9995	$y = 0.0126\ x + 0.00182$	0.169	86.5
Gallocatechin	0.9990	$y = 0.0108\ x + 0.00402$	0.880	113
Epigallocatechin	0.9994	$y = 0.00632\ x + -0.00825$	6.46	103
Catechin gallate	0.9992	$y = 0.00765\ x + -0.0002$	0.392	101
Gallocatechin gallate	0.9957	$y = 0.00733\ x + -0.00824$	1.87	120
Procyanidin B1	0.9992	$y = 1.05e{+}004\ x + 1.29e{+}003$	0.416	107
Procyanidin B2	0.9997	$y = 1.31e{+}004\ x + -399$	0.408	104
Quercetin	0.9995	$y = 5.67e{+}004\ x + -2.05e{+}004$	3.33	106
Rutin	0.9999	$y = 3.14e{+}004\ x + 234$	0.0428	87.7
		Range 3: 0.146 – 300 ng/mL		
Kaempferol	0.9970	$y = 5.14e{+}003\ x + -1.86e{+}004$	5.03	107
Procyanidin C1	0.9986	$y = 775\ x + -1.75e{+}003$	9.28	99.0
Procyanidin A2	0.9975	$y = 3.23e{+}003\ x + 1.88e{+}003$	4.80	102

ESI in negative ionization mode, and the turbo V source of the instrument was optimized using Flow Injection Analyses (FIA). The source parameters were the following: curtain gas 30 psig, nitrogen gas 1 50 psig, nitrogen gas 2 40 psig, temperature 500 °C, ionization spray operated at −4000 eV, and collision gas was set to High. Nitrogen was used as a source and collision gas. The data analysis was performed using Analyst software 1.7.1 from AB Sciex (Framingham, MA, USA).

2.4. Sampling and drying procedures

Branches of winter-dormant *Salix* spp. were collected in February 2022 on a willow farm in northern Jutland, Denmark. Branches were cut, preserved in plastic bags, and transported (1 h) to laboratory facilities at Aarhus University. The branches were debarked, and the bark was cut into smaller pieces and weighed. One part of the bark sample was freeze-dried (F), and the other was oven-dried (O). Before freeze-drying the bark, the sample was placed in a −80 °C freezer for 2 h. The freeze-dryer ScanVac CoolSafe (LaboGene A/S Lillerød, Denmark) operated at −40 °C for 72 h. After freeze-drying, the samples were placed in an exicator for 10 min before the final weighing. For oven-drying, the foil tray with bark was placed in the oven at 102 °C for 20 h. After the oven-drying, the sample was placed in an exicator for 10 min, and the first weighing was performed. The bark sample was placed in the oven at 102 °C for 2 h and a second weighing was performed. The dry matter (DM) was calculated for bark (freeze-dried 58.0% and oven-dried 55.7%). Further, the milling of the bark samples was performed in IKA TUBE-MILL 100 control, MT-40.100 at 25,000 rpm for appr. 2 min. The homogenous sample was afterward screened through a 500 μm sieve (Buch & Holm A/S, Herlev, Denmark). The bark samples were stored at −20 °C until further analyses.

2.5. Sample extraction

Bark samples (50 mg) were extracted with five different solvents (2 mL), hot water with initial temperature of 100 °C, water + 1% FA (v/v), 100% MeOH, 100% MeOH + 1% FA (v/v), and 100% MeOH + 1% HCl (v/v), Figure S1. All the extractions were performed at room temperature, and the bark samples were shaken for 1 hour, centrifuged at 1962 rcf for 10 min at 20 °C, and the supernatant was transferred to a new tube and stored at −80 °C. The extractions were performed in triplicates. Before LC-MS/MS analyses, extracted bark samples (25 µL of each extract) were diluted 100-fold in a working solvent of 5% ACN (v/v) and 1% FA (v/v) in water containing IS mix, reaching the final concentration of the standard curve.

2.6. Enzymatic hydrolysis of salicylic acid glycosides

Extracted bark samples (200 µL of each extract) were evaporated to dryness for 3–4 h using a ScanSpeed/ScanVac vacuum centrifuge from (LaboGene A/S Lillerød, Denmark) and reconstituted in 400 µL freshly dissolved β-glucuronidase (≥300,000 units/g solid)/sulphatase (≥10,000 units/g solid) (2 mg/mL in 50 mM sodium acetate buffer, pH 5) and incubated in a shaker at 37 °C for 19 h according to Smeds et al. [33] and Nørskov et al. [29]. After incubation, the enzyme was removed by adding 400 µL 1% FA in Water and centrifuged at 4 °C 29,700 rcf for 10 min. The supernatant was transferred to a new tube and diluted 1200-fold in the working solvent of 5% ACN (v/v), and 1% FA (v/v) in water containing IS mix. Samples were diluted an appropriate number of times depending on the final concentration of the standard curve.

2.7. Method validation

The guidelines for analytical method validation (European Medicines Agency) refer to several parameters to ensure the precision and trueness of the measurement [31]. The recovery of each analyte was assessed through spiking experiments measured on different days to calculate inter-day variability. The recoveries of the analytes were determined at concentrations fitting low (10 ng/mL), medium (20 ng/mL) and high (100 ng/mL) concentrations of the standard curve in the final sample and using five replicates per concentration. The sample preparation procedure for spiking experiment is shown in Fig. 2. The concentrations were calculated based on the stock 1 solution of standards accordingly to the dilutions and necessary spiking level dictated by the concentration levels of the compounds in the bark matrix. Unspiked bark extract (control) was used to subtract the background during the calculations. Spiked bark extract was diluted using the same dilution protocol for the samples to a final dilution of 100-fold (Fig. 2). Since unspiked bark extract already contained a high concentration of the number of analytes, some analytes were only possible to spike at one or two levels. Few analytes, catechin, procyanidin B1, and salicylic acid were present in the bark extract at high

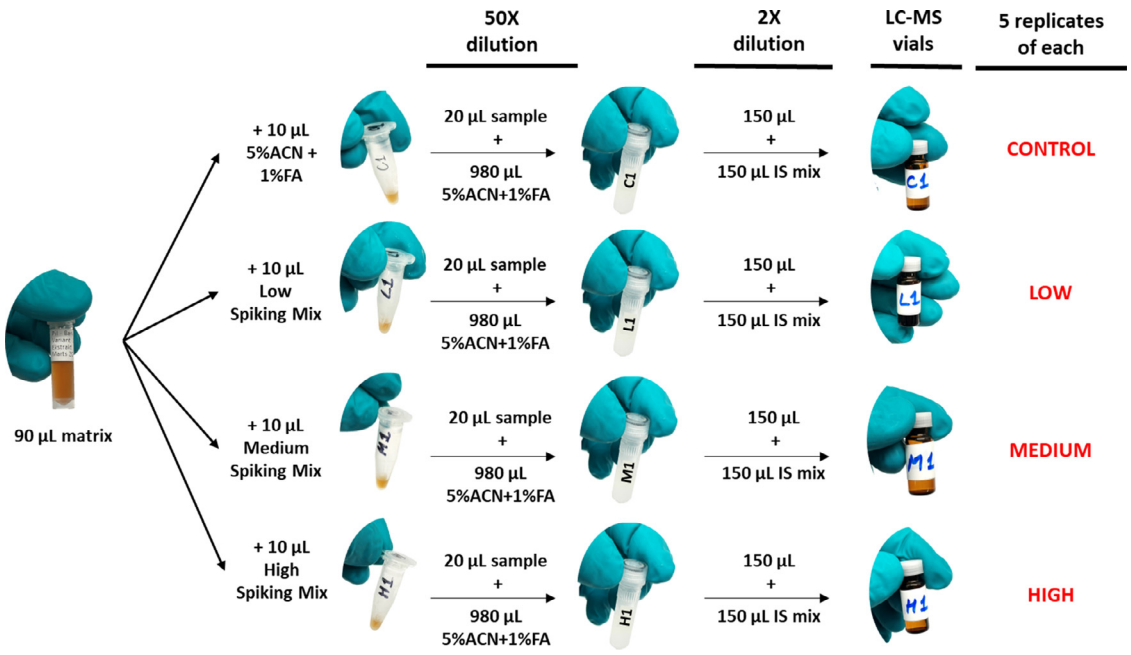

Fig. 2. Sample preparation steps for spiking experiment. Bark extract was spiked with using three levels of concentration (final concentration in sample: 10 ng/mL for low level; 20 ng/mL for medium level; 100 ng/mL for high level). The concentrations were calculated based on the stock 1 solution of standards accordingly to the dilutions and necessary spiking level. Spiked samples were prepared as regular samples following a dilution of 100-fold before LC-MS analysis.

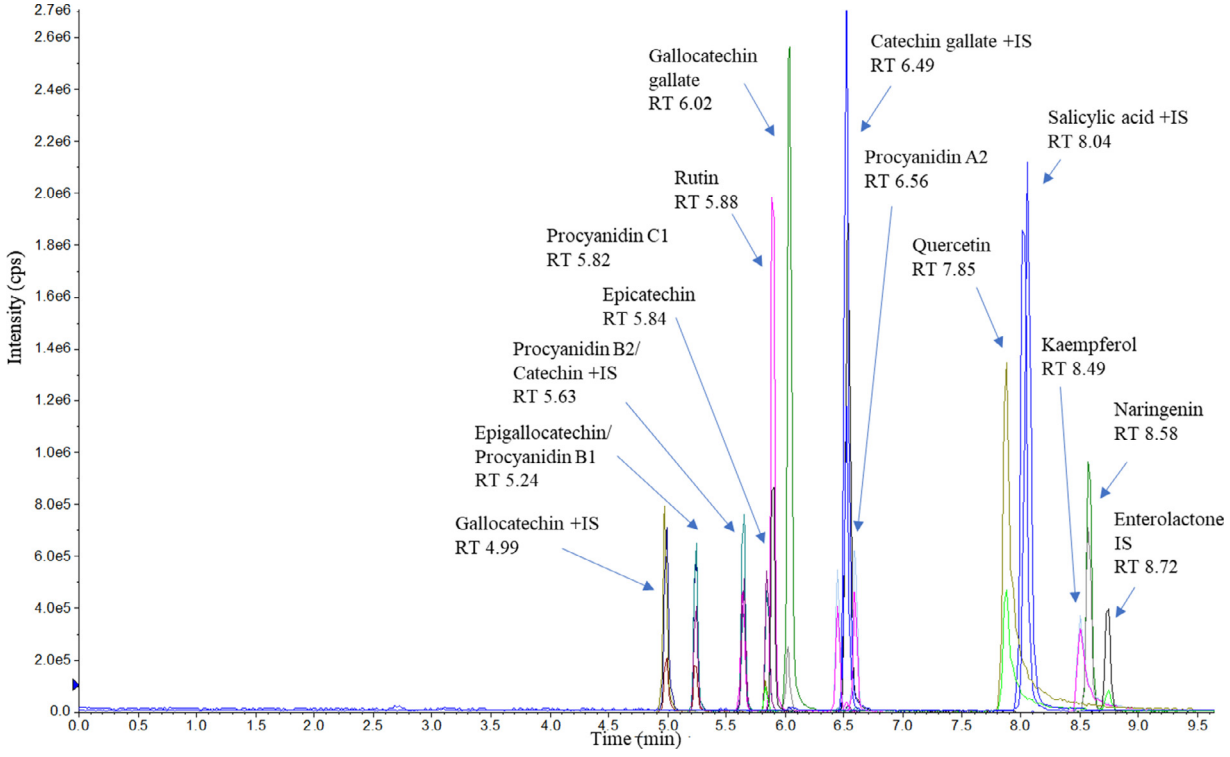

Fig. 3. MRM chromatogram of all standards and internal standards (IS) with the corresponding retention times (RT).

concentrations, for which spiking was not possible. Therefore, the precision of measurement was also accessed through the addition of IS mix containing five labelled standards, corresponding to the measured PCs eluting along the chromatographic gradient. IS mix represented labelled PCs, which eluted in the beginning (gallocatechin IS and catechin IS), middle (catechin gallate IS), and the end (salicylic acid IS and enterolactone IS) of the chromatogram, Fig. 3, representing elution of PCs throughout the gradient. Each sample and the standard curve contained an IS mix with matched concentrations. The analytical uncertainties due to sample injection, chromatographic shifts, and matrix effects were monitored through the IS mix during the chromatographic run and corrected during the calculation procedures. The IS mix was used as quality control, and the IS variation for analytes was monitored during the sample

analyses. Reference ST mix containing low, medium and high concentration of the analytes was used to calculate intra- and inter-batch variability and is presented in Table S2. The blank samples containing only solvent were injected to ensure no carry-over effect occurred.

2.8. Statistical analyses and calculations

The measured concentrations were used to calculate the final concentrations by accounting for the extraction, dilution factors, and the weight of the dry bark portion.

$$C_{PC} = \left((C_{measured} \times V_e \times DF)/W_{dry\ bark}\right)/1000000,$$

where C_{PC} is the final concentration of the PC (mg/g), $C_{measured}$ is the measured concentration (ng/mL), V_e is the extraction volume (mL), DF is the dilution factor, and $W_{dry\ bark}$ is the weight of dry bark portion used for the extraction. The final values were reported on a dry matter (DM) basis (mg/g). Further, the average and standard deviation of three extractions were calculated. The statistical analyses were conducted in SAS 9.4 (SAS Institute Inc., Cary, NC USA) using the general linear model procedure (PROC (GLM). The effects of the extraction solvents, dying procedure, and enzymatic hydrolysis were analyzed using the following linear model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the overall mean, α_i is the fixed effect of extraction solvent (i = S1, S2, S3, S4, S5), β_j is the fixed effect of drying (j = F, O), $\alpha\beta_{ij}$ is the interaction of solvent and drying, and ε_{ij} is the residual error component. Least squares mean estimates are reported. Multiple comparisons of interactions were adjusted using Tukey. Significance was declared at $P \leq 0.05$ and trend at $0.05 < P \leq 0.10$.

Recovery was calculated as a percentage of analyte recovered after the dilution procedure. The precision describes the percentage deviation of the mean from the actual value. Precision was calculated as relative standard deviation (RSD) of replicated measurements, with acceptance criteria that it should not deviate by more than ±15% at medium and high concentrations. Matrix effects were investigated by calculating the matrix factor (MF) for analytes and IS as the ratio between the peak area in the presence of matrix (sample) and the absence of matrix (standard curve). If the ratio was close to 100%, no ion suppression or enhancement had occurred. Inter-day variation was accessed through IS mix, and the coefficient of variation (CV) was calculated [31].

3. Results and discussion

3.1. LC-MS/MS method development and validation

3.1.1. Chromatography and tandem mass spectrometry

When applying reverse phase chromatography, the lipophilicity of the compounds, which is influenced by the number of aromatic rings, hydroxyl groups and glycosylation will define the elution from the column and, thereby, their RT. The chromatographic separation with the corresponding RTs of all the flavonoids and salicylic acid analyzed in this study is shown on the chromatogram of the standard curve in Fig. 3. Monomeric flavan-3-ols and procyanidins were the first compounds to elute. The high number of hydroxyl groups of procyanidins and sugar moiety of flavonoid glycoside, rutin, increased their hydrophilicity compared to flavonols (quercetin and kaempferol) and flavanone (naringenin). The RTs of the labelled standards matched the RTs of their corresponding non-labelled standard (Fig. 3). Due to the nature of PCs as lipophilic compounds containing benzene ring structures, phenyl-hexyl column has provided unique selectivity for aromatic compounds through their phenyl group and excellent retention through their extended hexyl hydrocarbon functional group. Although formic acid at 0.1% in water is most commonly used for the chromatographic elution of PCs [15–18], we have observed that the increase to 1% FA in water helped the binding capacity of the injected sample and also improved the chromatographic separation of close eluting isomers. In previous studies analyzing similar PCs, phenyl-hexyl stationary phase has been widely used [15–18,34].

Quantification of PCs was performed using MRM via collision-induced dissociation. The product ion spectra of PCs are shown in Figure S2. In terms of sensitivity, the negative ionization mode showed the best performance. The two most abundant fragments from the product ion spectra were chosen for quantification. The most sensitive transitions were set as quantifiers, and one more transition was used for verification (qualifier), as shown in Table S1. If one of the transitions was influenced by co-eluting compounds or other interferences during sample analyses, another transition was chosen as a quantifier. Other studies used similar transitions when measuring similar PCs. However, the intensities of fragment ions can be instrument-dependent [14,35].

3.1.2. Selectivity and sensitivity

Triple quadrupole mass spectrometers are well known for their high selectivity and sensitivity to differentiate analytes in the presence of other matrix components and detect them at low concentrations. Low background noise was observed for all the analytes, resulting in improved signal-to-noise ratio and increased sensitivity. The LLOQs and the linearity for all the analytes are presented in Table 1 and Fig. S4. The LLOQs varied between 0.0175 to 4.6 ng/mL, lowest for naringenin and highest for kaempferol and procyanidin C1. Selectivity of each PC and IS in the bark extract is presented in supplementary material, Fig. S3. We have previously experienced that a high number of isomers or interferences present in the sample can challenge the measurements of PCs in plant material. Although high dilution reduces the interference, isomers can cause the challenge for quantification. The highest number of additional peaks has been observed for procyanidins. However, in the case of procyanidin B1 and B2, the isomeric interferences have been well separated during the chromatographic run, as shown in Fig. S3. The separation of procyanidin A2 and C1 was challenged by the closely eluting isomeric interferences, decreasing the precision of the measurement. Chromatograms of all other PCs and their corresponding IS had high selectivity with this LC-MS/MS method, Fig. S3.

Our study achieved approximately ten times higher sensitivity than other studies [14,35]. However, variation in LLOQ has been observed and was as well reported in other studies [14,35–38]. Our experience is that the variation in the LLOQs among the PCs is closely linked to the ionization potential of each PC and their physicochemical properties, as well as the source-dependent parameters of the MS instrument. Compared to biological samples like plasma and urine or food products like milk, in which the concentration of PCs is low, the analysis of plant material is not challenged by the low concentration of PCs but by the concentration range [29,39,40]. However, good linearity has been observed for all the PCs in this method over the measured range of concentrations, Table 1 and Fig. S4.

3.1.3. Recovery, precision, and matrix effects

The recovery of the spiked analyte represents the amount of the analyte recovered after the sample preparation procedure. Depending on the complexity of the sample preparation procedure, analytes can be partly lost when clean-up and up-concentration procedures are applied. Therefore, the percentage of recovery demonstrates whether the sample preparation procedure is applicable for a particular number of analytes. Since *Salix spp*. contain high concentrations of PCs, no clean-up or up-concentration procedures were necessary when performing the quantification using triple quadrupole mass spectrometry. That diminishes the risk of analyte

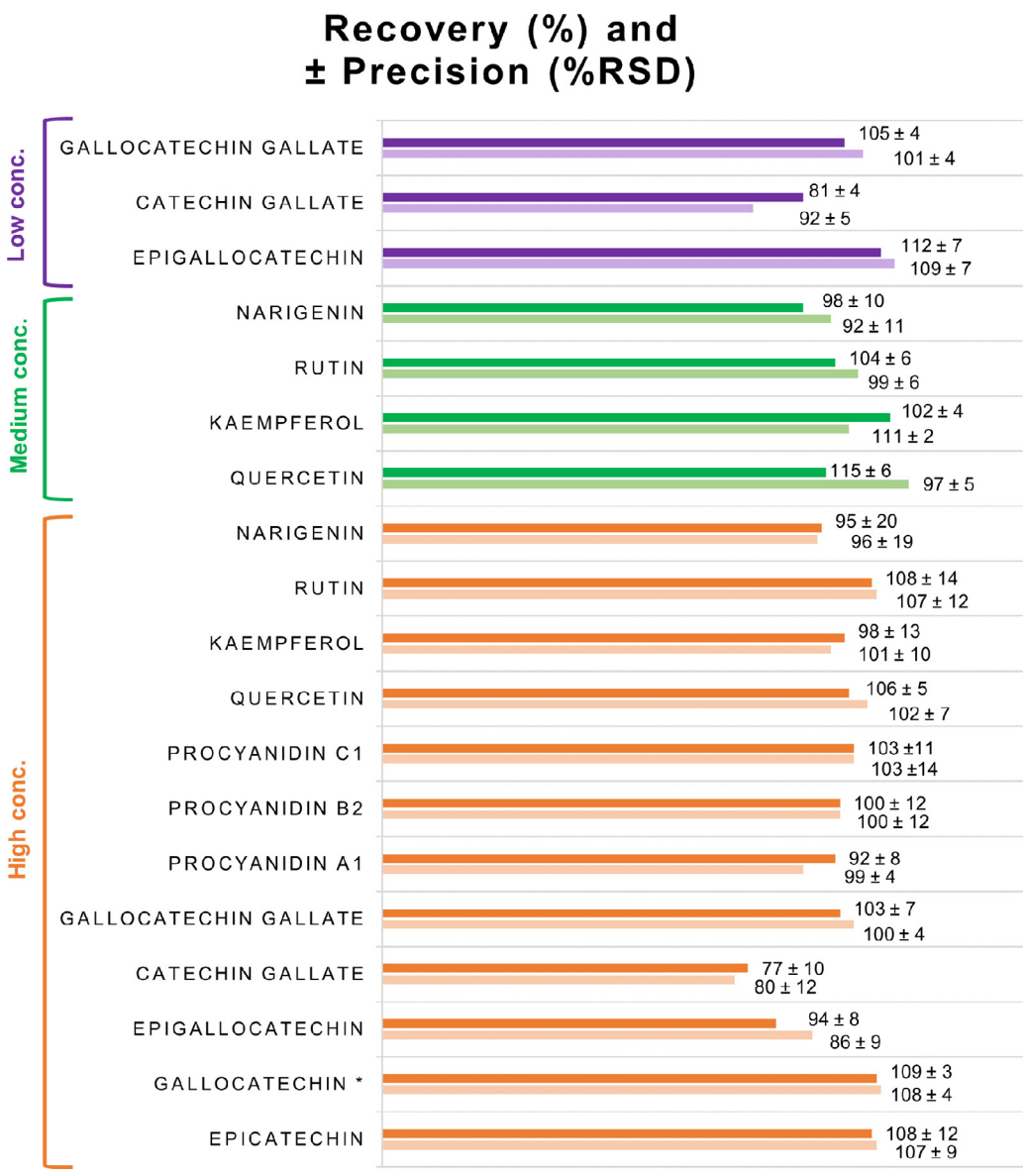

Fig. 4. Initial recovery (%, dark colored bars) and secondary recovery after 3 weeks at −80 °C (%, light colored bars) together with the corresponding precision (±RSD%) of the phenolic compounds ($n = 5$) spiked into extracted bark sample at low (10 ng/mL; purple), medium (20 ng/mL; green), and high concentration levels (100 ng/mL; orange); *gallocatechin ($n = 4$).

losses. Spiking experiments performed in this study, have shown high recovery and precision, of the measured PCs (Fig. 4). All the PC standards were spiked into extracted bark samples, $n = 5$, at the start of the dilution procedure using a working solvent of 5% ACN (v/v) and 1% FA (v/v) in water containing IS mix (Fig. 2). The challenge of spiking analytes in the sample matrix, which already contains varying concentrations of the measured PCs, is that spiking at only medium and high concentration levels is possible if matrix complexity matches the samples analyzed. Spiking with catechin, procyanidin B1, and salicylic acid was unsuccessful due to already high concentrations of these compounds in the bark extracts. Spiking at low levels was only possible for PCs that are not present in the extracted bark matrix. Spiking at low, medium, and high levels showed a high percentage of recovery for all the analytes tested, demonstrating the robustness of the entire procedure. Recoveries higher than 80% were calculated for all the PCs, although the recoveries for most PCs were close to 100%. The precision of replicated measurements was calculated to be within ±15%, varying between ±3% and ±14% depending on the analyte, except for naringenin at a high spiking level (Fig. 4). Inter-day variation based on spiking experiments was within 10%. Because high dilution factors (100-fold and 1200-fold) were applied, no matrix effects were observed, which is also demonstrated by the high recovery and precision of each analyte. Further, during sample analyses, matrix effects were monitored and calculated by Analyst (AB Sciex, Framingham, MA, USA) software using IS mix, which was spiked into each sample during dilution of the samples. Intra- and inter-batch variability was calculated to be within 11% for all the analytes varying between 1.7 and 10.9%, Table S2. Both, spiking experiment and IS spiking are reliable technique to demonstrate the validity of the LC-MS/MS method. Similar techniques have been used in other studies to demonstrate the validity of PC quantification [14,35–38,40]. The combination of a high number of ISs and high dilution without initial clean-up makes this LC-MS/MS powerful to quantify PCs in the bark of *Salix spp*. as well as other parts of the plant.

3.1.4. Stability of standards

Repeated analyses of the standard curve and IS mix in 5% ACN (v/v) and 1% FA (v/v) in water stored at −80 °C when not used over 3 months showed that the standards were stable for at least three months. Repeated analyses of the standard curve and IS mix in 5% ACN (v/v) and 1% FA (v/v) stored at room temperature over 48 h showed deterioration of gallocatechin and catechin after app. 24 h, therefore extracts and standards were stored at −80 °C when not in use.

3.2. The effect of drying procedures and extraction solvents

3.2.1. The effect of drying procedures

In this study, we have examined the effects of freeze-drying and oven-drying (oven-drying at 103 °C, which is traditionally used in many laboratories) and five widely used extraction solvents on the yield of flavonoids and salicylic acid from the bark of *Salix spp* (Fig. 5). The sum of the four monomeric flavan-3-ols will be referred to as the total yield of flavan-3-ols measured in this study, whereas the sum of the three polymeric flavan-3-ols will be referred to as the total yield of condensed tannins. The major effect was observed between freeze-drying and oven-drying. The freeze-drying procedure was better for preserving flavan-3-ols and condensed tannins than oven-drying, (Fig. 5a and 5b). Oven-drying at 103 °C of the bark samples generally resulted in the loss of total yield for both flavan-3-ols and condensed tannins. Oppositely, we did not observe any difference between freeze-drying and oven-drying at 103 °C for the yield of quercetin, naringenin and rutin, except when extracting with MeOH + 1% HCl (v/v), where the higher yield was for oven-drying (Fig. 5c). Salicylic acid was only quantified after enzymatic hydrolysis, and there was no difference between freeze-drying and oven-drying procedures (Fig. 5d).

Our results showed that drying procedures had different effects on the PCs depending on the subclass of flavonoids examined. Oven-drying at high temperatures (at 103 °C) decreased the yield of flavan-3-ols and condensed tannins. Previous studies corroborate these results. Julkunen-Tiitto found that increasing temperature (from 48 °C to 60 °C) decreased the total phenolic content of willow leaves [41]. More recently, different drying procedures were applied for flavonones, flavan-3-ols, and flavones. The study showed that air drying with the addition of heat (60 °C and 90 °C) decreased the concentration of most compounds [20]. Particularly catechin and the total condensed tannins were affected. Oppositely, freeze-drying at −30 °C was the effective way to preserve the PCs [20], similar to our results. Harbourne et al. did not find any significant difference between freeze-drying, oven-drying, and tray-drying at 30 °C and 70 °C and air-drying procedures on the concentration of condensed and hydrolyzable tannins, simple phenols, flavonoids, and total phenols, except for flavonoids when oven-dried at 70 °C in willow bark [19]. Interestingly we observed that two specific flavonoids, quercetin and naringenin, increased in concentrations when oven-drying was performed and independent of the solvent of extraction. We could speculate that the oven-drying helped degrade the matrix and release a proportion of matrix-bound compounds or promoted the breakdown of glycosidic bonds, releasing their respective aglycons. This effect was not observed for other compounds, such as salicylic acid, suggesting that salicylic acid glycoside was resistant to temperature. Other studies also conclude that total salicylic acid concentration in willow was not affected by drying treatment, and total amounts extracted from samples ranged from 2.3 to 2.5 mg/g [19]. The duration of exposure to light and temperature can be important factors to consider when developing protocols [42], as it has been shown that condensed tannins were unstable upon sunlight exposure and at room temperature three days after the extraction [12]. However, the samples in this experiment were extracted and preserved at −80 °C within one hour after extraction and kept at −80 °C when not in use. Therefore, we expect good preservation of the compounds.

3.2.2. The effect of extraction solvent

The total yield of the flavan-3-ols in the freeze-dried samples was highest with MeOH + 1% HCl (v/v) (6 mg/g DM) and lowest with hot water 3.7 mg/g DM (Fig. 4a). The extraction with MeOH + 1% HCl (v/v) also resulted in the highest total yield of condensed tannins, 3.5 mg/g DM, in the freeze-dried samples (Fig. 4b). For the flavan-3-ols, there were no differences between the total yield in the oven-dried samples when using MeOH with 1% FA (v/v) or 1% HCl (v/v). However, the lowest yield was obtained when extracting with hot water and MeOH, 3.05 mg/g DM and 3 mg/g DM, respectively (Fig. 4a). Extraction using MeOH + 1% HCl (v/v) significantly increased the yield of quercetin, naringenin and rutin in both freeze-died and oven-dried samples (Fig. 4c). MeOH + 1% HCl (v/v) resulted in a total yield of the three flavonoids of 0.74 mg/g DM in freeze-dried samples and 0.91 mg/g DM in oven-dried samples, whereas all the other solvents achieved a much lower yield between 0.2 and 0.4 mg/g DM (Fig. 4c). Solvent MeOH + 1% HCl (v/v) was also the best solvent for the extraction of salicylic acid in both freeze-dried and oven-dried samples (Fig. 4d).

Water and methanol, two widely used organic solvents have been used in this study. Previous studies have shown that the extraction of condensed tannins was more effective with hot water [19,24]. Our results showed that when compared to acidified room-temperature water, hot water had no significant impact on the extraction of condensed tannins or other PCs measured. We also did not observe any differences between the extraction with MeOH and MeOH + 1% FA (v/v). However, by acidifying MeOH with HCl, a much stronger acid compared to FA, the extraction yields were greatly improved for all the flavonoids and salicylic acid. Previous studies have shown similar findings [25,28]. Yield during extraction of PCs from grape pomace was highest with 1% HCl compared to 0 and 0.5% HCl [28]. The use acidified water (0.1%HCl) for extraction of total phenolic compounds, total flavonoid compounds, and total condensed tannins has shown to perform poorly in comparison to acidified aqueous methanol (70:30 MeOH:H_2O (v/v) + 0.1% HCl (v/v)) and acidified aqueous ethanol (70:30 MeOH:H_2O (v/v) + 0.1% HCl (v/v)) [25]. The extraction yields for total phenolics, and total condensed tannins were as follows: methanol > ethanol > water. However, for total flavonoid compounds, it was: ethanol > methanol = water [25]. Anthocyanins from grape pulp were also 20% more effectively extracted with MeOH than ethanol and 73% more effective than water extraction [43].

3.2.3. The effect of drying procedures and extraction solvent

The effects of drying procedures and extraction solvent on the yield of individual flavonoid are shown in Table 2. Drying procedures and extraction solvent significantly affected the yield of the individual flavonoids, epicatechin, gallocatechin, epigallocatechin, procyanidin B1 and quercetin. MeOH + 1% HCl (v/v) achieved the best yield for catechin, epicatechin, gallocatechin, epigallocatechin, and procyanidin B1 (only in the case of freeze-dried samples). In contrast, the extraction solvent affected less the yield of procyanidin C1 and procyanidin B2. For the extraction of catechin, the use of MeOH + 1% HCl (v/v) was comparable to the use of MeOH + 1% FA (v/v) and was best when compared to MeOH alone or hot water in the freeze-dried samples. For the oven-dried samples, concentrations of catechin were comparable across all extraction solvents. The uncertainty in the estimation of the mean remained low for the measurements of monomeric and polymeric flavan-3-ols as shown by the SEM values (Table 2).

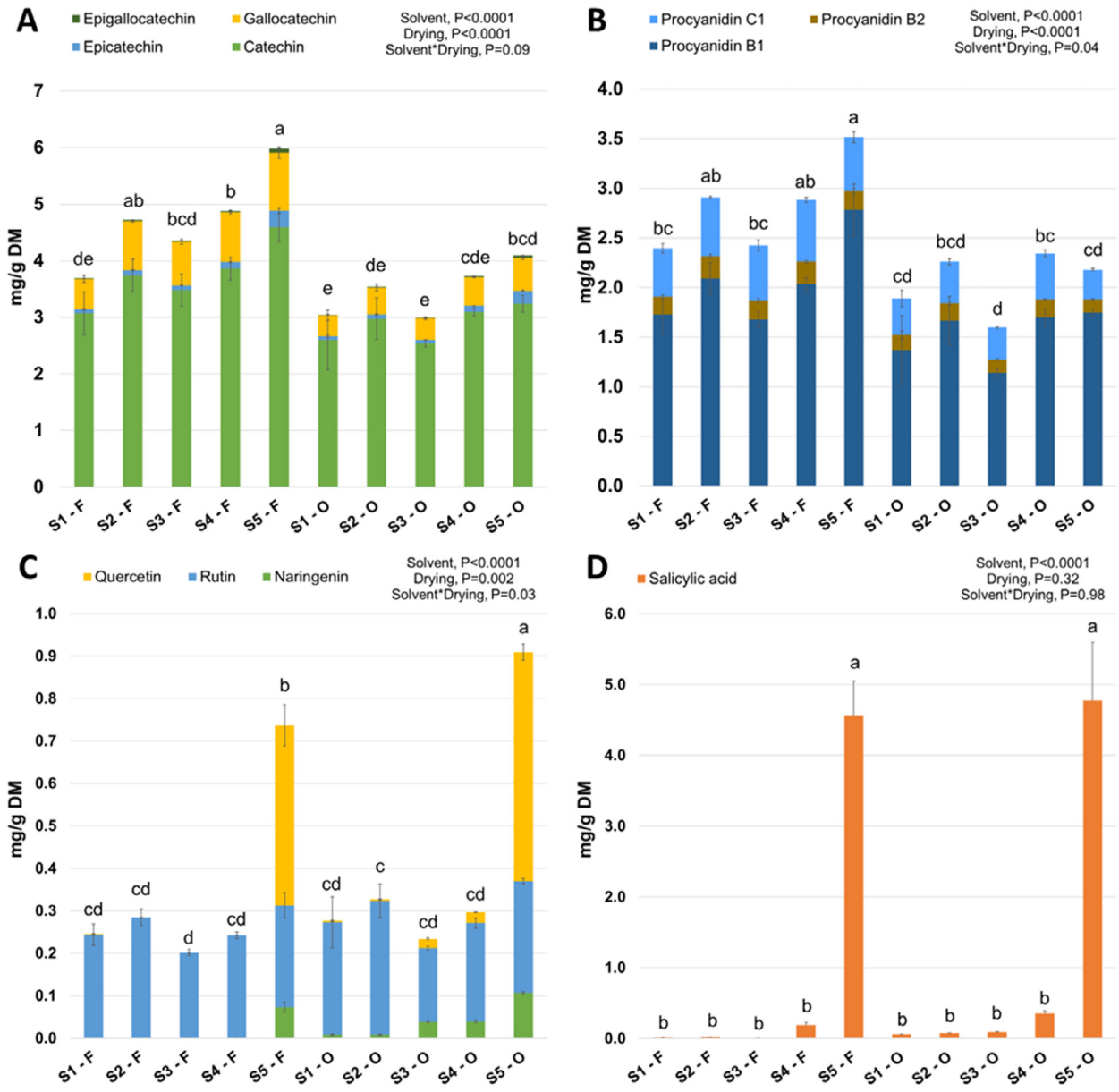

Fig. 5. Quantification of monomeric flavan-3-ols (A), condensed tannins (B), flavanol/flavonol glycoside and flavanone (C), and salicylic acid (D) in the bark of *Salix spp* and variation according to drying procedures and extraction solvents (S1 – Hot Water; S2 - Water + 1% FA (v/v); S3 – MeOH; S4 - MeOH + 1% FA (v/v); S5 - MeOH + 1% HCl (v/v); F – freeze-drying; O – oven-drying). Values with different superscript letters indicate a statistical difference in the total concentration of compounds ($p<0.05$). All extractions were performed in triplicates.

The results showed that the yield of condensed tannins was less affected by the extraction solvent. The choice of solvent influenced the extraction yields of quercetin and naringenin, with the highest yield obtained when using MeOH + 1% HCl (v/v) compared to other solvents. MeOH and MeOH + 1% FA (v/v) were comparable for the extraction of quercetin and naringenin, which had better yield compared to hot water or water + 1% FA (v/v). In the case of rutin, marginally higher extractions were observed with water + 1% FA (v/v) compared to MeOH alone. When MeOH was used in combination with 1% FA (v/v) or 1% HCl (v/v), concentrations of rutin were comparable. An increased concentration of quercetin and naringenin in the oven-dried samples compared to freeze-drying was observed, whereas the drying methods used had no impact on the concentrations of rutin in the samples. The extraction of quercetin and naringenin in freeze-dried samples was comparable between MeOH + 1% HCl (v/v), MeOH + 1% FA (v/v), and MeOH. However, MeOH + 1% HCl (v/v) performed better for the extraction of quercetin in oven-dried samples. Poor extraction with hot water, water + 1% FA (v/v), or simple MeOH for quercetin and naringin also increased the uncertainty in the estimation of the mean as shown by SEM values (Table 2).

Further, we observed that catechin was the most representative monomeric flavan-3-ol compound in the bark, whereas procyanidin B1 was the most abundant polymeric flavan-3-ol compound. Though previously reported in a flavanol glycoside form [7], kaempferol as aglycon was not detected in the bark of this *Salix spp*. The isomers of catechin and gallocatechin (i.e., epicatechin,

Table 2
Concentrations (mg/g DM) of individual (monomeric and polymeric flavan-3-ols), flavanols, and flavanone after freeze-drying (F) and oven-drying (O) procedures and five extractions solvents (S1 – Hot Water (100 °C); S2 - Water + 1% FA (v/v); S3 – MeOH; S4 - MeOH + 1% FA (v/v); S5 - MeOH + 1% HCl (v/v)). All the extractions were performed in triplicates and the mean of three extractions has been calculated (least square means). Values with different superscript letters indicate a statistical difference ($p<0.05$).

Compound /Drying method		Solvent					SEM	p-values		
		S1	S2	S3	S4	S5		Solvent	Drying	Solvent*Drying
					Monomeric flavan-3-ols					
Catechin	F	3.07	3.74	3.48	3.86	4.59	0.17	<0.0001	<0.0001	0.166
	O	2.6	2.98	2.54	3.1	3.24				
Epicatechin	F	0.073^{cd}	0.09^{cd}	0.08^{cd}	0.12^{c}	0.3^{a}	0.009	<0.0001	0.001	0.016
	O	0.06^{d}	0.08^{cd}	0.06^{d}	0.11^{c}	0.23^{b}				
Gallocatechin	F	0.54^{cd}	0.87^{ab}	0.77^{b}	0.88^{ab}	1.03^{a}	0.033	<0.0001	<0.0001	0.003
	O	0.37^{e}	0.48^{cde}	0.38^{de}	0.51^{cde}	0.58^{c}				
Epigallocatechin	F	0.009^{de}	0.016^{cd}	0.015^{cde}	0.018^{c}	0.071^{a}	0.002	<0.0001	<0.0001	<0.0001
	O	0.008^{e}	0.012^{cde}	0.009^{de}	0.015^{cde}	0.04^{b}				
					Polymeric flavan-3-ols					
Procyanidin B1	F	1.73^{bc}	2.09^{b}	1.68^{bc}	2.03^{b}	2.78^{a}	0.106	<0.0001	<0.0001	0.02
	O	1.37^{cd}	1.67^{bcd}	1.14^{d}	1.7^{bc}	1.75^{bc}				
Procyanidin B2	F	0.18	0.23	0.19	0.23	0.19	0.012	0.0006	<0.0001	0.621
	O	0.15	0.18	0.13	0.18	0.13				
Procyanidin C1	F	0.49	0.59	0.56	0.62	0.54	0.026	0.0002	<0.0001	0.124
	O	0.37	0.42	0.33	0.46	0.3				
					Flavanols					
Quercetin	F	0.001^{c}	–	–	–	0.424^{b}	0.012	<0.0001	0.0002	0.0002
	O	0.003^{c}	0.004^{c}	0.022^{c}	0.025^{c}	0.54^{a}				
Rutin	F	0.24	0.28	0.2	0.24	0.24	0.016	<0.0001	0.468	0.331
	O	0.26	0.31	0.17	0.23	0.26				
					Flavanone					
Naringenin	F	–	–	0.0004	0.001	0.073	0.003	<0.0001	<0.0001	0.618
	O	0.009	0.009	0.038	0.039	0.107				

epigallocatechin) were measured at lower concentrations than catechin and gallocatechin. Catechin gallate, gallocatechin gallate, and procyanidin A2 were not detected in the samples, irrespective of the drying procedures or extraction solvent.

PC extraction from plant material is critical for accurately quantifying these compounds. Typically, we refer to the quantification of extractable/soluble PCs as free compounds and soluble glycosides. However, PCs can also be present as bound to cell wall polymers through ester bonds as insoluble bound complexes and are less extractable when using organic solvents. Bound PCs can be liberated through acidic hydrolysis, alkaline hydrolysis, or both [24,29,44].

Besides the drying procedures and the extraction solvents, the analyses of PCs are complicated by the presence of PCs glycosides. Many PCs can be glycosylated with more than one glucose moiety, which complicates their quantification as glycosides due to the requirements for their corresponding standards. To be able to quantify the total concentration of a particular PC that can be found in both forms in the plant extract, further hydrolysis with beta-glucuronidase to release the aglycon is required [6,19]. Hydrolysis with *beta*-glucuronidase to release the aglycon for quantification has been previously shown to be an effective way to quantify the total concentration of specific PC glycosides [29]. Our results showed that enzymatic hydrolysis effectively quantifies salicylic acid, which could not be measured in the non-hydrolyzed samples. Salicylic acid was only detected after enzymatic hydrolysis in all the extraction solvents, although with the highest yield using MeOH + 1% HCl (v/v). Therefore, salicylic acid was mainly present as salicylic acid glycoside in the bark of *Salix spp*. Our results are confirmed by the qualitative analyses performed on the bark of *Salix spp*., where salicylates glycosides were detected [45].

4. Conclusions

The microLC-MS/MS method described was developed to quantify a wide range of PC concentrations and classes, flavan-3-ols, condensed tannins, flavonols, flavonol glycosides, flavanones, and salicylic acids in the bark of *Salix* spp. This microLC-MS/MS method allows the simultaneous quantification of 15 PCs using five stable isotope-labelled PCs combined with fast and simple sample preparation procedure, low consumption of chemicals due to microLC and high sensitivity and precision. Furthermore, we have shown the impact of sample preparation in terms of drying procedures. Freeze-drying was a superior method of flavan-3-ol preservation. We also examined the effects of five extraction solvents and acidification when performing classical solid-liquid extraction on the yield of individual PC, concluding that MeOH + 1% HCl (v/v) was the best extraction solvent compared to the others investigated in this study. Furthermore, we conclude that using enzymatic hydrolysis is crucial for correctly quantifying total concentrations of salicylic acid from willow bark and potentially other plant parts of *Salix* spp.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at …

Table S1. Compound-dependent parameters optimized by syringe infusion of pure standards. Declustering Potential (DP), Collision Energy (CE), and Cell Exit Potential (CEP).

Table S2. Intra- and inter-batch variation (±RSD%) of phenolic compounds (PCs) calculated from reference standard mix containing low, medium and high concentration of analytes. Intra-batch variation was calculated based on three replicated measure-

ments, $n = 3$. Inter-batch variation was calculated based on six/ten batches analyzed during 4 weeks/month, $n = 6$ and $n = 10$.

Figure S1. Different extraction solvents and the extraction color of willow bark.

Figure S2. Product ion spectra for the individual phenolic compound.

Figure S3. Extracted Ion Chromatograms of (XIC) of phenolic compounds (PCs) and corresponding internal standards (IS) spiked in the extracted bark matrix analyzed with the LC-MS method. The chromatograms show the selectivity of the method.

Figure S4. Calibration curves and ranges of the phenolic compound concentrations analyzed with the LC-MS method and quantified in the bark matrix. The figures contain the linear regression equation and r values for each phenolic compound.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mihai Victor Curtasu: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Natalja P. Nørskov:** Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

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