Evaluation of extraction methods for determination of phenolic compounds, organic acids and sugars in lingonberries (*Vaccinium vitis-idaea*)

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Abstract

Health benefits of lingonberries (*Vaccinium vitis-idaea*) have been linked to phenolic compounds, whereas organic acids and sugars are important for the taste of the berries. Contents of these compounds combined can be used to determine lingonberry quality. Extraction is a critical step in analyses, and many different methods have been used. The aim of the study was to evaluate different extraction methods and to determine if one single extraction method could be used to measure the content of phenolic compounds, organic acids, and sugars in lingonberries. Depending on extraction method and season, the concentrations of anthocyanins, flavonols, selected flavan-3-ols, and cinnamic acid containing compounds (CAC) in the lingonberries were 48-105, 14-32, 73-189, and 12-23 mg 100 g^{-1} fw, respectively. The organic acids (2.3-2.9 mg 100 g^{-1} fw) were mainly citric and quinic acid and the sugars (5.3-6.9 g 100 g) were glucose and fructose. 70% methanol was the best extraction solvent for anthocyanins, whereas 70% acetone was more efficient for flavan-3-ols. For extraction of CAC, flavonols, organic acids and sugars, 70% methanol and 70% acetone were equally efficient. Similar results were obtained from fresh and freeze-dried berries, but deviation between parallels was lower when extracting from freeze-dried berries. Considering both accuracy and time of preparation, extracting freeze-dried berries with 70% methanol was considered the best extraction method for analyzing compounds important for quality of lingonberries.

Keyword: freeze-drying, solvent, analysis, anthocyanins, flavonols, flavan-3-ols

INTRODUCTION

Lingonberries (*Vaccinium vitis-idaea*) contain a range of compounds contributing to health and sensory properties. Organic acids and sugars are the main contributors to taste (Viljanen et al., 2014), whereas phenolic compounds are associated with health promoting properties, such as prevention of urinary tract infection (Kontiokari et al., 2001) and improved glycemic response (Törrönen et al., 2012), in addition to cause astringency and bitterness of the berries (Kelanne et al., 2019; Peleg et al., 1999).

Phenolic compounds are most often analyzed by high performance liquid chromatography (HPLC) with different detectors (Aaby et al., 2013; Ek et al., 2006; Hokkanen et al., 2009; Hollands et al., 2017). Organic acids and sugars are analyzed using HPLC (Mikulic-Petkovsek et al., 2012; Woznicki et al., 2017) or gas chromatography (GC) (Kelanne et al., 2019; Marsol-Vall et al., 2021). Before analysis, the targeted compounds must be released from the plant material. Methanol and acetone, often in combination with water, are the most utilized extraction solvents for phenolic compounds (Escribano-Bailon and Santos-Buelga, 2003). For HPLC analysis, organic acids and sugars are most often analyzed directly in the diluted berry juice (Jensen et al., 2002; Mikulic-Petkovsek et al., 2012; Woznicki et al., 2017) while analysis by GC requires derivatization of the compounds (Kelanne et al., 2019; Marsol-Vall et al., 2021). Extractions of berries are performed on fresh or lyophilized berries.

As sample pretreatment is a time-consuming part of the analyses, it is preferred to have a common preparation method for both phenolic compounds, organic acids, and sugars. Furthermore, the method should be effective and accurate. The aim of this study was to evaluate different extraction methods and to determine if one single extraction method could

be used to measure the content of phenolic compounds, organic acids, and sugars in lingonberries.

MATERIALS AND METHODS

Plant material

Lingonberries (*V. vitis-idaea*) from the mid part of Norway (61.5°N, 10.1°E, ca. 600 m.a.s.l.) were collected from two growth seasons (year 1 and 2) and kept in the freezer (-18 °C) until further treatment.

Sample pre-treatment and extraction

The lingonberries were either extracted as frozen (fresh) or after freeze-drying (Gamma 1-16, Christ GmbH, Osterode am Harz, Germany). The fresh and dried material was extracted with water, 70% methanol (methanol/water, v/v 70/30) or 70% acetone (acetone/water, v/v 70/30) with methods previously used to extract polyphenols or sugars/acids from fruits and berries (Aaby et al., 2013; Aaby et al., 2012; Davik et al., 2020; Hollands et al., 2017; Woznicki et al., 2017) (Table 1).

The dried samples were homogenized for 15 s in a small blade mill (OBH Nordica, Groupe SEB, France), prior to addition of extraction solvent and sonication for 10 min (Ultrasonic Cleaner, VWR International, Leuven, Malaysia). The "fresh" material was partly thawed and homogenized in a food processor prior to addition of extraction solvent and homogenization in a Polytron PT3100 homogenizer (Kinematica AG, Littau, Switzerland) at 28,000 rpm for 30 s followed by sonication for 5 min. The water and methanol extracts were centrifuged at 39200 *g* for 10 min at 20 °C (Avanti J-26 XP Centrifuge, Beckman Coulter, USA) and the acetone extracts were centrifuged at 1500 *g* for 10 min at 20 °C (Heraus Multifuge 4KR, Kendro Laboratory Products GmbH, Hanau, Germany). The supernatant was collected, and the insoluble plant material re-extracted with the extraction solvent. The supernatants were pooled, and the volume made up to 10-50 mL (Table 1). The extractions were performed at ambient temperature (20-22 °C), except one extraction with 70% methanol performed at 60 °C (Hollands et al., 2017).

Table 1. Extraction conditions.

Sample name	Material	Weight (g)	Extraction solvent	Temp. (°C)	Extraction volumes (mL)	Total volume (mL)	Ref.[a]
Fresh_H_2O	Fresh	5.00	Water	20-22	15	15	a, b, c
Dry_H_2O	Dried	0.50	Water	20-22	15	15	a, b, c
Fresh_MeOH	Fresh	3.00	70% methanol[b]	20-22	2 x 10	25	d
Dry_MeOH	Dried	0.40	70% methanol	20-22	2 x 5	20	d
Dry_MeOH_60	Dried	1.00	70% methanol	60	50	50	e
Fresh_Acetone	Fresh	5.00	70% acetone[c]	20-22	2 x 10	10 [d]	b, d, f
Dry_Acetone	Dried	0.40	70% acetone	20-22	2 x 5	10 [d]	b, f

[a]a (Woznicki et al., 2017), b (Aaby et al., 2013); c (Mikulic-Petkovsek et al., 2012), d (Davik et al., 2020), e (Hollands et al., 2017), f (Aaby et al., 2012).

[b]100% methanol was used in the first extraction.

[c]100% acetone was used in the first extraction.

[d]Acetone was evaporated from pooled extracts by a nitrogen flow at 37 °C (Sample concentrator, Techne, Stone, Staffordshire, UK). The volume of the extracts was made up to 10 mL with water.

The extractions were performed in triplicate. Concentrations were converted to per g fresh weight (fw) based on the dry weight (14.5%) of the berries. The extracts were stored at – 20 °C until analysis within four months. The extracts were filtered through Millex HA 0.45 µm filters (Millipore Corp., Billerica, MA, USA) prior to analysis.

Analysis of phenolic compounds

Polyphenols were analysed on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 4 °C, a diode array detector (DAD), and an MSD XCT ion trap mass spectrometer fitted with an electrospray ionization interface as previously described (Aaby et al., 2013). Separation was performed on a Synergi 4 µm MAX RP C12 column (250 mm × 2.0 mm i.d.) equipped with a 5 µm C12 guard column (4.0 mm × 2.0 mm i.d.), both from Phenomenex (Torrance, CA, USA) at 40 °C. The mobile phases were formic acid/water (2/98, v/v) and acetonitrile and the solvent flow rate was 0.25 mL min^{-1}. Identification of phenolic compounds were based on their retention times, UV-vis (220–600 nm) and mass spectra in positive and negative mode and comparison with literature (Ek et al., 2006; Hokkanen et al., 2009) and authentic standards when available. The compounds were quantified by external standards. The anthocyanins were quantified as equivalents of cyanidin-3-galacoside (at 520 nm), flavonols as rutin (at 360 nm), CAC as chlorogenic acid (at 320 nm), and flavan-3-ols as catechin (at 280 nm).

Analysis of organic acids and sugars

Samples (20 µL) were injected on an Agilent 1100 Series HPLC (Agilent Technologies) equipped with an autosampler cooled to 4 °C, a DAD, and a refractometer index (RI) detector (Model 132; Gilson, Villiers-le-Bel, France). Separation was performed on a Rezex ROA-Organic acid H+ (8%) column (300 × 7.8 mm; Phenomenex, Torrance, CA, USA) at 45 °C with mobile phase 7.2 mmol L^{-1} H_2SO_4 and flow rate 0.5 mL min^{-1} as previously described (Woznicki et al., 2017). The sugars were quantified with RI detection and the organic acids with DAD at 210 nm. Identification of the compounds was based on comparison and spiking with authentic standards. Quantification was achieved by use of external standard curves.

RESULTS AND DISCUSSION

Anthocyanins

Five anthocyanins with major compounds cyanidin-3-galactoside (82-86%), cyanidin-3-arabinoside (9-12%), and cyanidin-3-glucoside (3-5%) were detected in the berries (Figure 1A). The anthocyanin composition was in accordance with previous reports (Andersen, 1985; Lee and Finn, 2012). Total anthocyanin concentration in the extracts varied from 48 to 105 mg 100 g^{-1} fw (Table 2). The anthocyanins were most efficiently extracted from freeze-dried berries with 70% methanol, and poorly extracted with water. Standard deviation between extraction parallels were in general higher for fresh berries than for dried material.

Flavonols

Composition of flavonols, consisting mainly of glycosides of quercetin (Figure 1B), was in accordance with previous findings in lingonberries (Ek et al., 2006; Marsol-Vall et al., 2021). The concentrations of flavonols were 14 - 32 mg 100 g^{-1} fw, with the lowest amounts in the aqueous extracts (Table 2, Figure 1B). Standard deviation between extraction parallels were higher for fresh berries than for dried material.

Flavan-3-ols

Monomers, dimers, and trimers of flavan-3-ols were detected, and is represented in Figure 1C. Due to co-elution with other phenolic compounds and low extinction coefficients, quantification of flavan-3-ols was difficult, and may be one of the reason for the differing results compared with concentration of flavan-3-ols in lingonberry juice (Marsol-Vall et al., 2021). Concentrations of flavan-3-ols were slightly higher in the acetone extracts, compared with water and methanol extracts, except for the methanol extract of fresh berries from year 2 (Table 2). 70% acetone is commonly used as extraction solvent for flavan-3-ols (Escribano-Bailon and Santos-Buelga, 2003; Kylli et al., 2011).

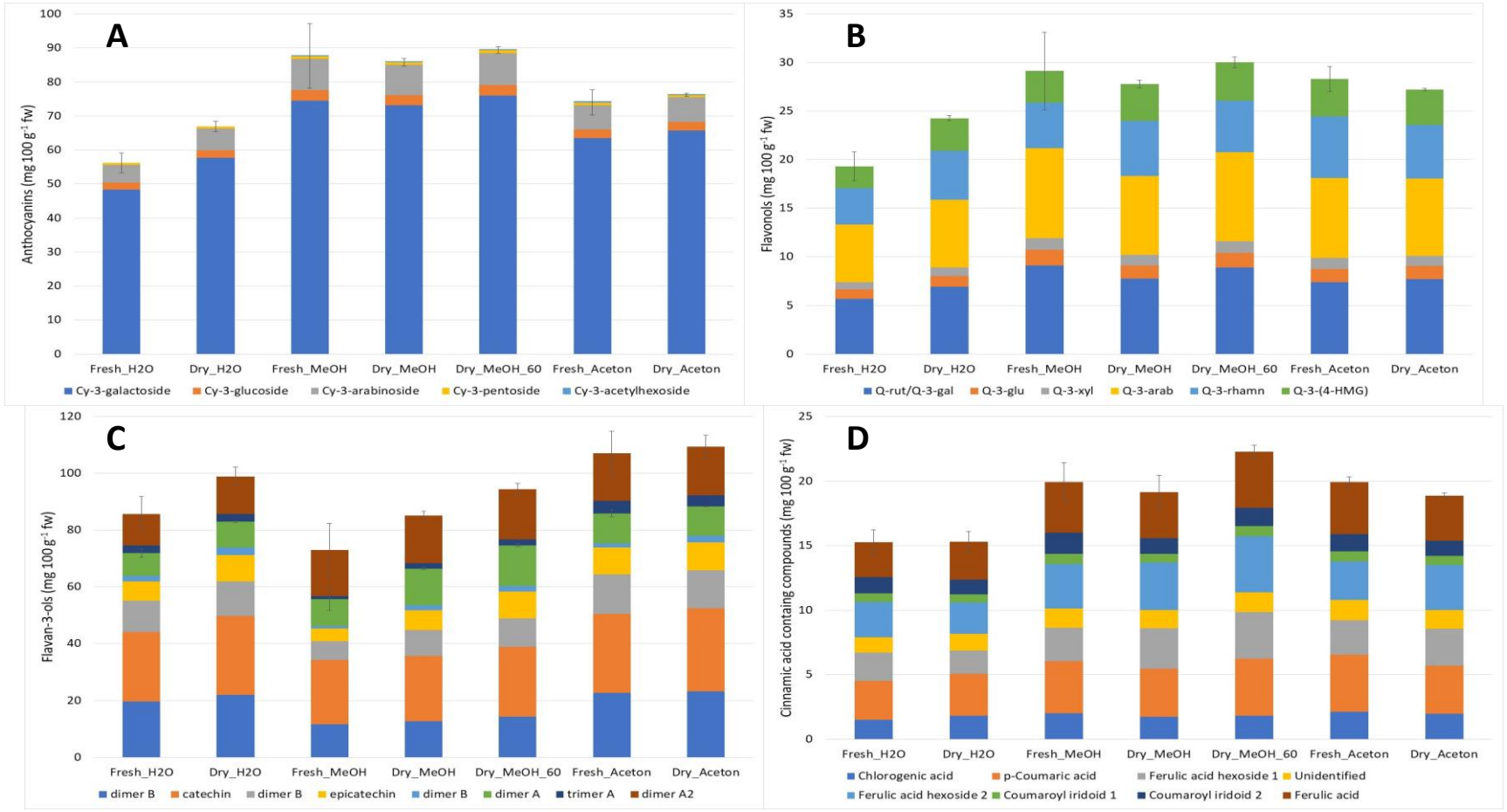

Figure 1. Composition of phenolic compounds in extracts of lingonberries from year 1. A, anthocyanins. Cy = cyanidin. B, flavonols. Q = quercetin, rut = rutinoside, gal = galactoside, glu = glucoside, xyl = xyloside, arab = arabinoside, rhamn = rhamnoside, HMG = 3-hydroxy-3-methylglutaroyl. C, flavan-3-ols. D, cinnamic acid containing compounds (CAC).

Table 2. Total concentrations of anthocyanins, flavonols, cinnamic acid containing compounds (CAC), flavan-3-ols, sugars, and organic acids lingonberries extracted as fresh (frozen) or freeze-dried with different extraction solvents.

	Anthocyanins (mg 100 g^{-1} fw)		Flavonols (mg 100 g^{-1} fw)		CAC (mg 100 g^{-1} fw)		Flavan-3-ols (mg 100 g^{-1} fw)		Sugars (g 100 g^{-1} fw)		Acids (g 100 g^{-1} fw)	
Sample	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 1**	**Year 2**
Fresh_H_2O	56 ± 3	48 ± 3	20 ± 1	14 ± 2	16 ± 1	12 ± 1	86 ± 6	101 ± 5	5.5 ± 0.2	6.5 ± 0.1	2.3 ± 0.0	2.3 ± 0.0
Dry_H_2O	67 ± 2	74 ± 2	26 ± 0	20 ± 0	16 ± 1	12 ± 0	99 ± 3	138 ± 1	6.8 ± 0.1	6.8 ± 0.1	2.9 ± 0.0	2.9 ± 0.0
Fresh_MeOH	88 ± 10	87 ± 14	31 ± 4	30 ± 6	20 ± 2	18 ± 2	73 ± 9	184 ± 16	6.3 ± 0.2	6.9 ± 0.3	2.7 ± 0.0	2.9 ± 0.0
Dry_MeOH	86 ± 1	105 ± 1	29 ± 0	23 ± 1	20 ± 1	14 ± 1	85 ± 1	120 ± 6	6.6 ± 0.2	5.5 ± 0.1	2.8 ± 0.1	2.7 ± 0.0
Dry_MeOH_60	90 ± 1	102 ± 1	32 ± 1	23 ± 1	23 ± 0	15 ± 1	94 ± 2	129 ± 1	6.6 ± 0.1	5.6 ± 0.1	2.8 ± 0.0	2.8 ± 0.1
Fresh_Acetone	75 ± 4	76 ± 8	30 ± 1	25 ± 5	21 ± 0	14 ± 1	107 ± 8	162 ± 9	6.7 ± 0.3	6.5 ± 0.3	2.7 ± 0.0	2.5 ± 0.2
Dry_Acetone	77 ± 0	86 ± 8	29 ± 0	21 ± 2	20 ± 0	14 ± 1	109 ± 4	162 ± 12	6.5 ± 0.1	5.3 ± 0.2	2.7 ± 0.0	2.6 ± 0.1

Cinnamic acid containing compounds (CAC)

Chlorogenic acid, *p*-coumaric acid, ferulic acid and two ferulic acid hexosides were the most abundant CAC in the extracts (Figure 1D). Two coumaroyl iridoides, previously found in both lingonberry leaves and juice (Hokkanen et al., 2009; Jensen et al., 2002), were also detected in the berries. The percentage distribution of the compounds was different in year 2 than in year 1 (results not shown) and the total concentration of CAC was lower in the berries from the second season (Table 2). In berries from both years, however, the lowest concentrations of CAC were found in the water extracts. The standard deviations between extraction parallels were similar from dried and fresh berries.

Sugars

The lingonberry extracts contained 5.3 – 6.9 g 100 g^{-1} fw sugar (Table 2). The sugars were glucose (48-53%) and fructose (47-50%) (results not shown). The methanol and acetone extracts from year 2 also contained sucrose (ca. 4%). Sucrose can be converted to fructose and glucose by sucrase (invertase), and the absence of sucrose in the berries stored for a longer period (year 1) and in water extracts of berries from year 2, indicates that sucrose had been hydrolyzed in these samples. The concentration and composition of sugars are in accordance with previous findings in lingonberries (Mikulic-Petkovsek et al., 2012). There were lower deviations between extraction parallels of dried berries and the aqueous extract of dry berries had stable, high concentration of sugars over the two years. However, the decomposition of sucrose could be avoided with extraction with an organic solvent.

Organic acids

The lingonberry extracts contained 2.3 – 2.9 g 100 g^{-1} fw acids, with the lowest concentration in aqueous extracts of fresh berries (Table 2). The standard deviations between extraction parallels were in general low. The total acid concentration was in accordance with previous findings in lingonberries (Jensen et al., 2002; Mikulic-Petkovsek et al., 2012; Viljakainen et al., 2002). The acids present were citric acid (58-63 %), quinic acid (37-41%), and shikimic and fumaric acid, ca 0.1% each (results not shown). The composition was in line with findings in lingonberries from Sweden (Jensen et al., 2002) and Finland (Kelanne et al., 2019). However, others have reported the presence of malic and tartaric acid, and not quinic acid, in lingonberries (Mikulic-Petkovsek et al., 2012; Viljakainen et al., 2002)

CONCLUSION

Composition and concentration of phenolic compounds, sugars, and organic acids in Norwegian lingonberries from two growth seasons were in accordance with previous reports. Similar results were obtained from fresh and freeze-dried berries, but deviation between parallels was lower when extracting from freeze-dried berries. Methanol was the best extraction solvent for anthocyanins, whereas acetone was more efficient for flavan-3-ols. For extraction of cinnamic acid containing compounds (CAC), flavonols, organic acids and sugars, methanol and acetone were equally efficient. Organic acids and sugars could also be efficiently extracted with water from freeze-dried berries. However, the decomposition of sucrose could be avoided with extraction with an organic solvent. Considering both efficacy, accuracy, and time of preparation, extracting freeze-dried berries with methanol was considered the best extraction method for phenolic compounds, sugars, and organic acids, and could be used as the single extraction method for compounds important for quality of lingonberries.

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