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Multiple reaction monitoring for identification and quantification of oligosaccharides in legumes using a triple quadrupole mass spectrometer

Virginia Prieto-Santiago [a], María del Mar Cavia [a], Francisco J. Barba [b], Sara R. Alonso-Torre [a], Celia Carrillo [a,*]

[a] Nutrición y Bromatología, Facultad de Ciencias, Universidad de Burgos, E-09001 Burgos, Spain
[b] Nutrition and Food Science Area, Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine, Faculty of Pharmacy, Universitat de València, Avda Vicent Andrés Estellés, s/n. 46100, Burjassot, València, Spain

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ABSTRACT

Raffinose family oligosaccharides are non-digestible compounds considered as dietary prebiotics with health-related properties. Hence, it is important to develop highly specific methods for their determination. An analytical method is developed in this study for oligosaccharide identification and quantification using liquid chromatography-tandem mass spectrometry equipped with a triple quadrupole analyser operating in Multiple Reaction Monitoring mode. Raffinose, stachyose and verbascose are separated in a 10-minute run and the method is validated over a broad concentration range, showing good linearity, accuracy, precision and high sensitivity. A low-cost, short eco-friendly procedure for oligosaccharide extraction from legumes, with a high recovery rate extraction, good repeatability and reproducibility is also proposed. No plant-matrix effects were demonstrated. The method applied to the screening of 28 different legumes revealed species-related traits for oligosaccharide distribution, highlighting *Pisum sativum* (9.22 g/100 g) as the richest source of these prebiotics and its suitability as a functional food ingredient.

1. Introduction

Raffinose Family Oligosaccharides (RFOs), or α-galactosides, are low-molecular weight, non-reducing and non-structural carbohydrates that are widely distributed throughout the plant kingdom, are soluble in water and water-alcohol solutions. RFOs are characterized by the formation of α-1,6 glycosidic linkages between a sucrose molecule and galactosyl residues. Chemically, α-galactosides are considered to be sucrose derivatives since they are made up of combinations of D-galactose units linked to the D-glucose moieties group of the sucrose molecule, giving rise to a number of oligosaccharides (Dey, 1980). The main α-galactosides are trisaccharide raffinose (α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside), tetrasaccharide stachyose (α-D-galactopyranosyl-(1 → 6)-α-Dgalactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside) and pentasaccharide verbascose (α-D-galactopyranosyl-(1 → 6)-[α-D-galactopyranosyl-(1 → 6)]2-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside).

RFOs are Non-Digestible Oligosaccharides (NDOs). The human digestive tract produces no α-galactosidase, the enzyme that could otherwise assist the digestion and subsequent metabolism of RFOs, by cleaving the α-galactosyl moieties from oligosaccharides (De Fátima et al., 2005). These compounds consequently resist hydrolysis by digestive enzymes, thus avoiding their absorption in the upper part of the gastrointestinal tract, and they therefore pass into the large bowel where they promote the growth of *Bifidobacterium* and *Lactobacillus* (Roberfroid, 2002). These bacteria belong to a group of saprophytic bacteria, which have proven positive effects on the host. Some reported health benefits include antimicrobial, anticarcinogenic and antimutagenic properties, reduced levels of serum cholesterol, and improvement in lactose tolerance, as well as a lower epidemiological incidence of allergies (Ouwehand, Salminen, & Isolauri, 2002; Roberfroid, 2002). As a consequence of their microbiota growth-promoting effect, and subsequent health-related properties, raffinose, stachyose and verbascose have been considered as dietary prebiotics (Rastall, 2013). Therefore, once the potential health benefits of RFOs have been demonstrated, it is necessary to have highly specific qualitative and quantitative methods to accurately determine this family of compounds.

Several analytical methods have been reported for the determination

* Corresponding author.
E-mail address: ccarrillo@ubu.es (C. Carrillo).

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of oligosaccharides, among which chromatographic techniques are the most commonly used for the separation of these compounds (Lee, 1987). Gas chromatography requires sample derivatization as oligosaccharides are non-volatile (Molnár-Perl, 2000). High Performance Liquid Chromatography (HPLC), considered the standard technique for this type of analyte (Gupta, Jain, Gill, & Gupta, 2012), entails the separation of analytes on the basis of their affinity within the stationary phase and the mobile phase and the consequent migration of each species through the column.

Oligosaccharides have been detected by different analytical techniques coupled to chromatographic separation. The lack of any chromophore or fluorophore group in oligosaccharide structures prevent their direct detection by ultraviolet and fluorescence detectors (Grembecka, Baran, Błażewicz, Fijałek, & Szefer, 2014; Lee, 1987). Although HPLC coupled with Refractive Index Detectors (RID), Evaporative Light Scattering Detectors (ELSD), and Pulsed Amperometric Detectors (PAD) are commonly used in carbohydrate analysis, drawbacks related either with poor sensitivity, specificity, selectivity and even a lack of compatibility with gradient elution have been reported (Cataldi et al., 2000; Knudsen, 1986; Lee, 1987; Martens & Frankenberger, 1991; Wong, Baggett, & Rye, 2003).

Mass Spectrometry (MS) offers a significant advantage over the aforementioned detectors, due to its specificity and sensitivity and the structural information that it provides on the analyte. The triple quadrupole tandem mass spectrometer (QQQ-MS/MS) gives information on the characteristic production of the molecular ion with several operational modes that offer different types of quantification. The Selected Reaction Monitoring (SRM) mode involves the determination of an analyte by monitoring a specific precursor ion and a specific product ion generated by Collision-Induced Dissociation (CID), which enhances specificity and reduces background noise and interference (Yang et al., 2002). The improvement of the signal-to-noise ratio accurately determines low abundant compounds. Using the same concept as SRM, Multiple Reaction Monitoring (MRM) mode follows multiple fragmentation events for potentially multiple analytes during an analytical run (Guo, Chen, Liu, Zhu, & Yao, 2012). Two or more product ions can be acquired for a certain analyte for both quantification and verification purposes. QQQ-MS/MS in MRM mode therefore represents a very sensitive method that can detect and quantify small molecules through the screening of specific molecular ion-to-product ion transitions (Sherwood et al., 2009), so that the interference of overlapping HPLC peaks may be avoided.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) is a robust tool for enabling the simultaneous identification, structural characterization and quantification of oligosaccharides. A Quadrupole Ion Trap Mass Spectrometer (QIT-MS) equipped with an ElectroSpray Interface (ESI) operating in the positive ion mode has been used for the quantification and structural characterization of raffinose family oligosaccharides in *Casuarina glauca* plant tissue (Jorge, Florêncio, Ribeiro-Barros, & Antonio, 2017) while *Lupinus albus* oligosaccharides have been determined by Ion Trap (IT) mass spectrometer (Antonio et al., 2008). The Selected Ion Monitoring (SIM) mode acquisition has been applied both for the determination and the quantification of oligosaccharides in legumes using an LTQ/Orbitrap XL hybrid mass spectrometer (Fan, Zang, & Xing, 2015) as well as in complex mixtures using a quadrupole mass detector (LC-Q) (Brokl, Hernández-Hernández, Soria, & Sanz, 2011). Oligosaccharides in wheat flour have been characterised and quantified by means of a Q-TOF (Liu & Rochfort, 2015), although MRM mode quantification of oligosaccharide with a triple quadrupole mass spectrometer has yet to be reported.

Therefore, taking into account the current background regarding the identification and quantification of oligosaccharides, this study aims to develop and to validate an analytical method for the accurate quantification of oligosaccharides through liquid chromatography tandem mass spectrometry with a triple quadrupole analyser (LC-MS/MS (QQQ)) operating in MRM scan mode, in order to improve both the detection and the quantitation of these compounds within a short analysis time. Applied to a legume food matrix, which is the main source of these compounds in the diet, the optimized method proposed in our study was used to screen 28 different varieties to determine their oligosaccharide content.

2. Material and methods

2.1. Chemicals and reagents

Raffinose and stachyose standards were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Verbascose was obtained from Megazyme (Bray, Ireland). HPLC grade acetonitrile, formic acid and ethanol were used (VWR/DBH Chemicals, Barcelona, Spain).

2.2. Experimental set up

Firstly, a LC-MS/MS (QQQ) analytical method operating in MRM mode for the simultaneous assessment of oligosaccharides (raffinose, stachyose and verbascose) was developed. Method validation involved an assessment of the linearity, limit of detection, limit of quantification, precision and accuracy with pure standards.

Secondly, the analytical method was applied to the measurement of oligosaccharides in legumes. To do so, different extraction methods were compared and the selected approach was also validated by means of (i) repeatability and reproducibility of the extraction method, (ii) recovery efficiency and (iii) matrix effects. Finally, a screening of the oligosaccharide content of 28 different legumes was performed according to the method proposed in this study.

2.3. MRM scan mode optimization. Analytical method validation

2.3.1. Preparation of standard solutions

Stock solutions of each individual standard oligosaccharide were prepared in ultrapure water (Millipore, Molsheim, France) (5000 mg/L). These stock solutions were used to prepare a mixture containing 500 mg/L of the standards. Dilutions of either pure or mixed standards were carried out using mobile phase.

2.3.2. Chromatography and mass spectrometry

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis was performed in an Agilent 1260 Infinity system (including degasser, quaternary pump and temperature controlled autosampler kept at 4 °C) coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an AJS-ESI (Agilent Jet Stream ionization source). A Polaris NH_2 (250x4.6 mm, 5 μm) column was used for the analyte separation process. The mobile phase consisted of ultrapure water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid at a ratio of 40:60 (v:v). The flow rate was 0.6 mL/min, with no elution gradient applied and the injection volume was set at 5 μL.

The MS was operated in Multiple Reaction Monitoring (MRM) mode. MRM analysis was used to monitor the transitions from precursor ions to dominant product ions. Two specific transitions were used to determine each compound, with the most intense transition used for the quantification (quantifier transition) and the second used for confirmation purposes (qualifier transition). The optimized source parameters were: sheath gas temperature 350 °C; sheath gas flow 8 L/min; nebulizer 30 psi; capillary 4000 V; nozzle voltage 0 V; drying gas temperature 340 °C; drying gas flow 7 L/min. Source conditions were optimized using Source Optimizer Software (version B.08.00).

2.3.3. Linear range and sensitivity

The linearity of the method was established by linear regression analysis on raw data using $1/\times$ as the weighting factor. This analysis was performed using three replicates of 13 calibration standard levels within the range 0.005–10 ng (amount on column). The peak area of the

quantifier transition of each oligosaccharide was plotted against the concentration of each corresponding standard, while the linear range was assessed by fitting the data, starting at a moderately low concentration to a straight line by linear regression analysis. Extreme concentrations were removed until R^2 was equal to or higher than 0.99.

A signal-to-noise approach should only be applied to analytical procedures that emit baseline noise. Since noise is essentially negligible in MRM mode, the Limit Of Detection (LOD) and the Limit Of Quantification (LOQ) were estimated using the slope and the standard deviation of a specific calibration curve containing the analyte in the range of LOD/LOQ (Validation of Analytical Procedures, 2005).

2.3.4. Method accuracy and precision

Accuracy and precision were determined by testing oligosaccharide standards at low, medium, and high concentrations (0.1, 1 and 10 ng – amount on column) within the calibration range. Accuracy represents the deviation between the actual measured concentration and the nominal amount injected (Validation of Analytical Procedures, 2005). Three replicates were prepared for each standard and concentration and calculated as the percentage of recovery according to Eq. (1). The coefficient of variation (CV) was calculated for each mean percentage recovery.

$$Recovery\ (\%) = \frac{Concentration\ \ measured}{Nominal\ \ concentration} \times 100 \quad (1)$$

The precision of the method was evaluated through repeatability (intra-day precision) and reproducibility (inter-day precision) (Validation of Analytical Procedures, 2005). Repeatability was calculated by determining the CV of the mean concentration obtained from the injection of three replicates of the standard solutions over a single day. For reproducibility, the same standards were injected over five different days and the CV of the mean concentration was calculated.

2.4. Extraction of oligosaccharides from legumes

2.4.1. Samples

Twenty-eight dried legumes were obtained from a local market in Burgos (Spain) (May 2019) two varieties of the genus *Cicer*, one variety of the genus *Glycine*, six varieties of the genus *Lens*, one of the genus *Lupinus*, twelve varieties of the genus *Phaseolus*, one of the genus *Pisum*, one of the genus *Vicia* and four varieties of the genus *Vigna*.

A reference sample, prepared by pooling all legumes in equal proportions, was used for the validation of the extraction method.

2.4.2. Comparison of extraction methods

Extraction efficiency of the three target oligosaccharides (raffinose, stachyose and verbascose) from the legumes was assessed using two different solvents, ultrapure water and ethanol (80%) and a multi-cycle extraction protocol. Briefly, 0.1 g of the powdered reference sample were treated with 5 mL of solvent and submitted to vigorous shaking for 10 min, followed by centrifugation (4 °C, 10 min, 15,763*g*) to collect the supernatant. This optimized extraction protocol was carried out three times. The concentrations of the three oligosaccharides recovered after each independent extraction cycle were quantified by MRM scan mode after using 0.2 μm filtration (polypropylene membrane filters).

2.4.3. Extraction repeatability and reproducibility

Five independent extractions of the reference sample over three different days were performed, to determine the repeatability and reproducibility of the optimized extraction method. The subsequent MRM quantification of the three oligosaccharides was then performed. The repeatability of the extraction method was calculated through the CV of the mean concentration obtained from the injection of the five replicates of the extraction performed over a single day. For reproducibility, the extractions performed over three different days were injected and the CV of the mean concentration was calculated.

2.4.4. Recovery assay

Extraction efficiency was determined by means of the recovery assessment, as previously indicated (Saar, Gerostamoulos, Drummer, & Beyer, 2009). To determine recovery, the reference powdered sample was spiked with three different concentrations of raffinose, stachyose and verbascose pure standards and it subsequently underwent the entire optimized extraction procedure described above (Section 2.4.2). Experiments were performed in quintuplicate and the recovery was calculated according to eq. (2).

$$Recovery\ (\%) = \frac{Total\ \ mass\ \ in\ \ spiked\ \ sample - mass\ \ in\ \ non\ \ spiked\ \ sample}{mass\ \ spiked} x\ 100 \quad (2)$$

2.4.5. Matrix effects

The effect of a plant matrix on oligosaccharide MRM quantification was evaluated through a post-extraction spiked procedure (Armah, Ferruzzi, & Gletsu-Miller, 2020). An oligosaccharide extract from the powdered reference sample was firstly obtained according to the optimized extraction method described above (Section 2.4.2). Three different concentrations of raffinose, stachyose and verbascose pure standards were then added to the prepared extract. Matrix effect assay was performed in quintuplicate and calculated according to Eq. (3).

$$Matrix\ \ effect\ \ (\%) = \frac{Total\ \ mass\ \ in\ \ spiked\ \ sample - mass\ \ in\ \ non\ \ spiked\ \ sample}{mass\ \ spiked} x\ 100 \quad (3)$$

2.5. Statistical analyses

Data were analyzed using Statgraphics Centurion XVI software (StatPoint Technologies, Inc., USA). The significance of differences between mean values was determined by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test, with a value of $P < 0.05$ considered as statistically significant. Both the applicability and the reproducibility of the method were confirmed by the mean sum of squares for variety and replication obtained from a general linear model applied to ANOVA. Principal Component Analysis (PCA) was performed on the correlation matrix and two principal components were extracted.

3. Results and discussion

3.1. Optimization of MRM quantification mode. Analytical method validation

3.1.1. Optimization of ion source parameters

A standard solution of each oligosaccharide was directly injected into the triple quadrupole and several parameters were tested to maximize the signal intensity for the three compounds, in order to optimize the parameters of the ion source. **Table S1** (supplementary material) shows the range assessed for each parameter and the optimized value selected and used throughout in subsequent analyses.

3.1.2. Selection of MRM transitions

Each compound was individually infused into the triple quadrupole, in order to optimize MRM conditions, with the predominant adducts identified while operating the MS instrument in full scan mode. Using product ion scan mode, fragment ions were then detected and the four most intense ones were automatically (*Optimizer* software; version B.08.00) selected. The fragmentor and the collision energy were optimized for each transition by applying a 100–280 V and a 5–65 eV ramp, respectively. In this study, both positive and negative ionization modes were initially investigated. It was observed that oligosaccharides were able to ionize in both positive and negative mode, in close agreement with the results of Liu and Rochfort (2015). However, the abundance of product ions found in negative mode were too low for verbascose. Therefore, the positive ionization mode was selected for subsequent experiments.

The positive ionization mode yielded sodiated molecules $[M + Na]^+$ (raffinose m/z = 527, stachyose m/z = 689, verbascose m/z = 851) (Fig. 1**A–C**), which were selected as precursor ions for the MS product ion scan. The fragmentation pattern previously described for the three oligosaccharides (Domon & Costello, 1988; Jorge et al., 2017) corresponded with our findings (Fig. 1**D–F**). The product ion spectrum of the sodiated trisaccharide raffinose $[M + Na]^+$ at m/z 527 produced an intense ion at m/z 365, formed by cleavage of the glycosidic bond and the loss of one hexose moiety ($C_6H_{10}O_5$), and an ion at m/z 203, formed by the loss of another hexose moiety. In the case of the sodiated tetrasaccharide stachyose $[M + Na]^+$ at m/z 689, the main product ions were observed at m/z 527 and at m/z 365, as a result of subsequent losses on one hexose moiety. Finally, the MS product ion spectrum of the sodiated pentasaccharide verbascose ($[M + Na]^+$ at m/z 851) yielded a high abundant ion at m/z 689 (formed by cleavage of the glycosidic bond and the loss of one hexose moiety) and a second most abundant ion at m/z 527 (formed by the loss of another hexose moiety).

The selected transitions used for each compound are shown in **Table S2** (supplementary material). The quantification required one MRM transition and the other (referred to as a qualifier ion) was used for confirmation purposes. This strategy is in agreement with the basic practice used for MRM-based quantification on a triple quadrupole, ensuring better specificity (Magi, Scapolla, Di Carro, & Liscio, 2010). Whenever an interfering matrix ion is of the same m/z ratio as the analyte of interest and produces a fragment at the same m/z ratio as the first product ion, then the absence of the qualifier ion confirms that this signal is an interference. The first product ion is used for analyte quantification, while the presence of the second product ion is useful for confirming the analyte of interest and discriminates against any interfering ions. Both MRM transitions for each analyte are particularly useful when working with quantifying analytes in a complex matrix. The optimized transition for each specific compound represents a robust assay that serves to quantify oligosaccharide content in complex mixtures through MRM analysis.

Oligosaccharide separation with NH_2 columns has been successfully reported in various studies using mobile phases mainly acetonitrile and water (Oboh et al., 2000; Raja, Agasimani, Varadharajan, & Ram, 2016). Then, the optimization of LC separation using mobile phases consisting of different water–acetonitrile ratios and using a solution with a mixture of authentic standard oligosaccharides was carried out. Fig. 1**G** shows the chromatogram in MRM mode. Under the optimized chromatographic conditions, the three oligosaccharides were detectable with clearly differentiated elution in a 10-minute run, with raffinose, stachyose and verbascose at 8.02, 8.80 and 9.66 min, respectively, according to the elution profile described in the literature (Antonio et al., 2008; Han & Baik, 2006).

3.1.3. Linearity and sensitivity of the method

Table 1A summarizes the results of the regression analysis concerning the linearity of the method over the quantification range. The LC-MS/MS (QQQ) method showed an excellent linearity over the entire concentration range (0.1–10 ng) for the three oligosaccharides, with determination coefficients (R^2) > 0.99. As the response of the detector was linear over two orders of magnitude, target oligosaccharides could be quantitatively analyzed in different types of samples differing in the content of these compounds.

The on-column LOQ and LOD ranged from 0.007 to 0.015 ng and from 0.002 to 0.005 ng, respectively, with the limits of detection and quantification indicating the high sensitivity of the analytical method. As far as it can be ascertained, these values are within the lowest concentrations of detection reported so far. Our results were within the range reported by Jorge et al. (2017) when they used electrospray quadrupole ion trap mass spectrometry (0.003–0.005 ng) and showed higher sensitivities than most previously reported values. Liu and Rochfort (2015) reported a LOD of 0.25 ng for raffinose with a Q-TOF mass spectrometer. Fan et al. (2015) found a LOD of 0.1 μg/ml for raffinose and stachyose by mass spectrometry operated in SIM mode. Gangola, Jaiswal, Khedikar, and Chibbar (2014) and Kotha, Finley, and Luthria (2020) used a PAD detector and reached detection limits of 4, 5–15 and 3–11 ng for raffinose, stachyose and verbascose, respectively. Significantly lower sensitivities were reported for RI detectors, with LOD of 170 μg/ml and 1380 μg/ml for raffinose and stachyose, respectively (Frias, Hedley, Price, Fenwick, & Vidal-Valverde, 1994).

3.1.4. Method accuracy and precision

The results of accuracy and intra- and inter-day method precision are shown in Table 1B. The mean recovery percentages were 96.2% for raffinose, 98.8% for stachyose and 103.9% for verbascose, showing good recovery with a CV lower than 1.6% for the 3 oligosaccharides, which highlights the high accuracy of the method. The data were also characterized by their relatively high precision. All oligosaccharide CV values for intra- and inter-day precisions were lower than 1.6% and 7.2%, respectively (Commission decision of 12August implementing Council Directive96/23/EC concerning the performance of analytical methods and the interpretation of results2000, 2002).

3.2. Application of the method to legumes

3.2.1. Oligosaccharide extraction from legumes. Comparison of extraction methods

The extraction efficiencies of oligosaccharides under the different conditions that were assessed are shown in Fig. 2. The first extraction cycle recovered a significantly higher content of raffinose, stachyose and verbascose (21.3%, 20.7% and 55.2% higher content, respectively) when water was used as extraction solvent. In contrast, during the second and third cycles, ethanol (80%) achieved a significant higher oligosaccharide extraction (3–15 times higher). When the recoveries along the three extraction cycles were combined, water was the most efficient solvent in extracting verbascose (11.5% higher content of verbascose extracted). Furthermore, although no significant differences between the efficiencies of both solvents were observed for raffinose, stachyose and total oligosaccharides extraction, a trend toward higher recoveries when water was used could still be found. Taking these results together and in view of the fact that water is considered to be more

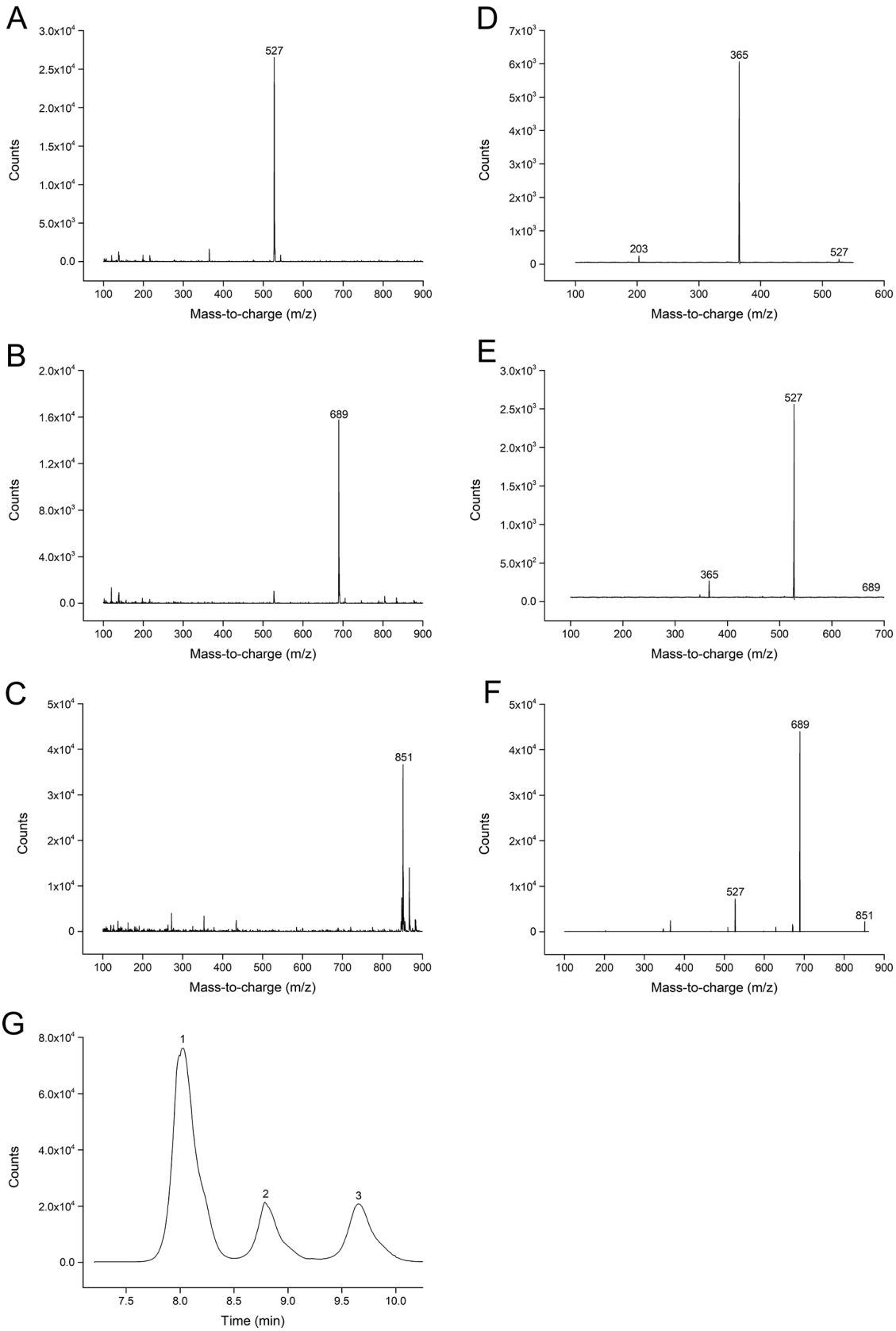

Fig. 1. Typical MS and MS/MS spectrum (positive ionization mode) of raffinose (A and D, respectively), stachyose (B and E, respectively) and verbascose (C and F, respectively). Multiple Response Mode (MRM) chromatogram (G). Peak asignment in panel G refers to raffinose (1), stachyose (2) and verbascose (3).

Table 1A
Linearity and sensitivity of the method.

	Linearity							Sensitivity	
	R^2	*Linear range (ng)*	*Slope*		*Intercept*		*CV (%)*	*LOQ (ng)*	*LOD (ng)*
			value	p-value	value	p-value			
Raffinose	0.9986	0.1–10	158,181	0.0000	1544	0.2542	3.06	0.007	0.002
Stachyose	0.9992	0.1–10	100,054	0.0000	975	0.0197	3.79	0.015	0.005
Verbascose	0.9997	0.1–10	60,128	0.0000	−1384	0.0012	4.72	0.008	0.003

Values expressed in ng refer to on-column amounts. LOD: limit of detection. LOQ: limit of quantification. CV: coefficient of variation. CV was calculated from the mean value of the response factor (ratio between peak area and analyte quantity) (n = 3).

Table 1B
Accuracy and precision of the method over the calibration range.

	AOC (ng)	Accuracy		Precision (CV %)	
		Recovery (%)	*CV (%)*	*Intra-day*	*Inter-day*
Raffinose	0.1	89.53	1.44	1.44	3.32
	1	102.43	1.18	0.81	7.11
	10	96.61	0.48	0.48	1.89
Stachyose	0.1	99.68	0.78	0.78	4.73
	1	98.04	0.83	0.52	4.34
	10	98.71	0.14	0.14	2.31
Verbascose	0.1	115.59	1.58	1.58	5.61
	1	94.81	1.53	0.04	5.56
	10	101.44	0.16	0.16	1.56

AOC: amount on column. CV: coefficient of variation (n = 3).

sustainable than organic solvents, water was therefore selected as a solvent for the extraction procedure.

When comparing the extraction efficiencies of the three cycles applied, the oligosaccharides recovered in the third cycle accounted for less than 1% of total oligosaccharide extraction, with two cycles needed for almost complete extraction of the target compounds (99.6%). The two-cycle-water extraction procedure was therefore selected for the extraction of oligosaccharides in legumes and used for the subsequent analysis.

Although hydroethanolic mixture is widely used for the extraction of polar metabolites from plant samples (Aylangan, Ic, & Ozyardimci, 2017; Han & Baik, 2006; Oboh et al., 2000; Raja et al., 2016; Tosh et al., 2013), our results showed similar extraction efficiency (even higher for verbascose) when water was used. These findings are in keeping with results reported for wheat flour (Liu & Rochfort, 2015) and in line with other studies that also used water for the extraction of these compounds

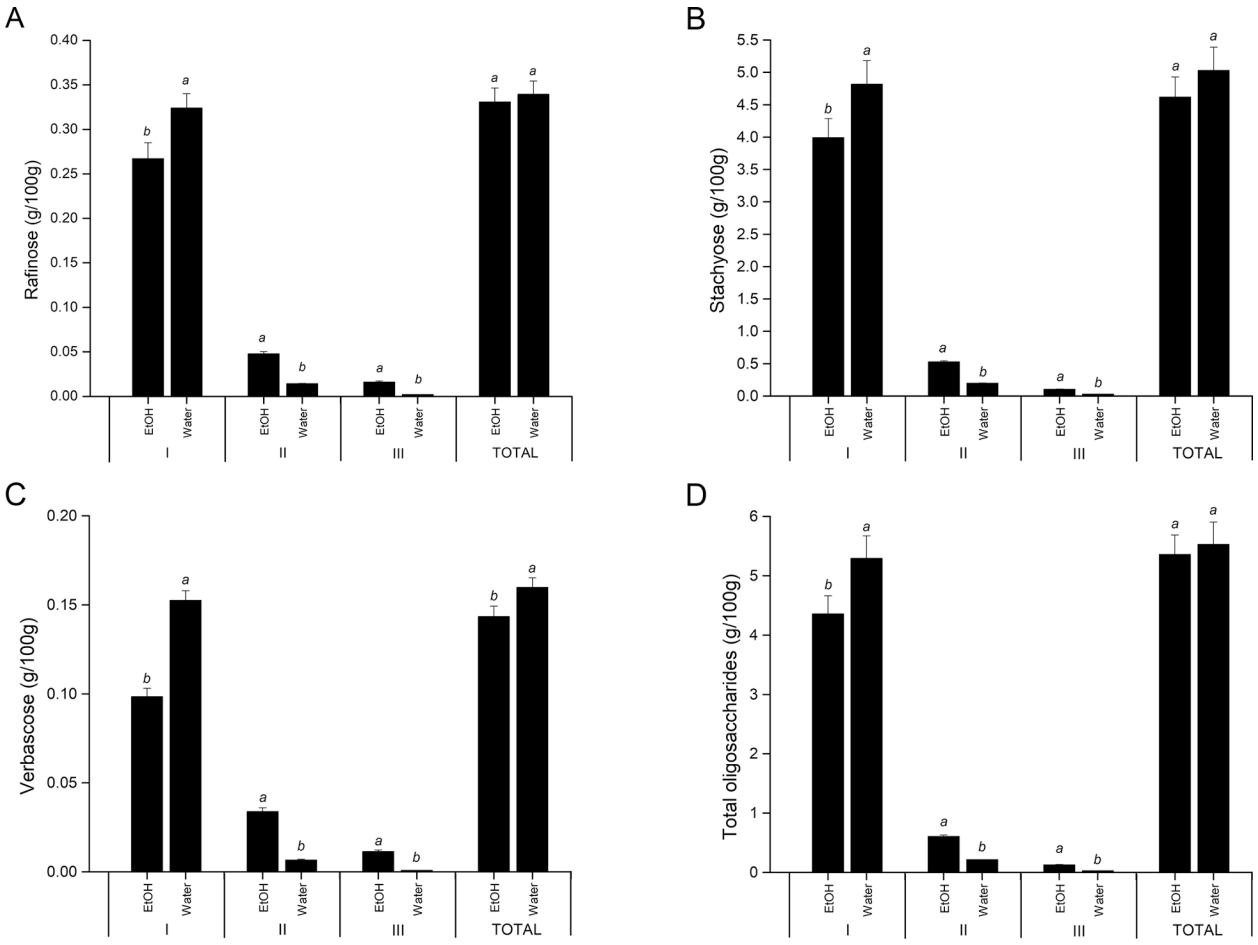

Fig. 2. Extraction of raffinose (A), stachyose (B), verbascose (C) and total oligosaccharides (D) using water or etanol (80%) during three subsequent extraction cycles (I, II and III). Concentrations expressed as mean ± SD (n = 3).

Table 2
Repeatability and reproducibility of the extraction method. Recovery and matrix effects.

	Repeatability CV (%)	Reproducibility CV (%)	Recovery (%) (CV %)			Matrix effects (%) (CV %)		
			x	*2x*	*4x*	*x*	*2x*	*4x*
Raffinose	1.28	6.23	91.47 (2.37)	94.83 (4.34)	97.70 (2.06)	101.90 (2.27)	101.87 (2.41)	96.35 (1.00)
Stachyose	1.54	8.37	106.40 (3.69)	100.34 (5.18)	103.47 (1.05)	97.67 (3.62)	97.13 (1.87)	91.69 (0.58)
Verbascose	3.44	7.54	111.57 (2.21)	92.73 (1.57)	96.88 (4.38)	91.31 (1.36)	93.58 (1.56)	93.01 (0.75)

CV: coefficient of variation (n = 5). Values in brackets include the CV for the recovery and matrix effect assay. "x" (spiked amounts) refers to (ng, amount on column) 0.2, 1 and 0.1 for raffinose, stachyose and verbascose, respectively, in the recovery assay. "x" refers to 1 ng (amount on coumn) in the matrix effect assay.

in legumes (Fan et al., 2015).

3.2.2. Extraction reproducibility and repeatability

The analysis of repeatability (intra-day) and reproducibility (inter-day) of the extraction procedure presented CV values that were lower than 3.5% and 8.5%, respectively, for the three oligosaccharides, indicating the acceptance of the extraction method proposed (Table 2).

3.2.3. Recovery of oligosaccharides from the extraction

The mean recovery percentages for raffinose, stachyose and verbascose were 94.7%, 103.4% and 100.4%, respectively (Table 2), showing that the proposed extraction method was acceptable for the three target oligosaccharides without significant losses occurring during the extraction procedure. Our results are within the range of previously reported data using water as the extraction solvent (Fan et al., 2015).

3.2.4. Matrix effects

The definition of matrix effects relates to the effects of non-target matrix compounds on the ionization efficiency of compounds of interest. If ion suppression or ion enhancement occurs, then matrix effects are usually observed (Trufelli, Palma, Famiglini, & Cappiello, 2011). In positive polarity, numerous compounds such as proteins, peptides, amino acids and salts can form positively charged ions, thereby contributing to higher degrees of ion suppression. Nevertheless, the extent of any ionization suppression will be analyte-dependent. Management and investigation of matrix effects is therefore important for the validation of a method and its implementation, as inaccurate measurements of the target compounds may otherwise occur (Chiu et al., 2010).

Different methods have been proposed in the literature to assess the presence or absence of matrix effects during a LC–MS method development. In the present study, a post-extraction addition method consisting of the addition of known concentrations of the standards solutions to an oligosaccharide extract was used. Following this approach, we tested the effects of a legume plant matrix on raffinose, stachyose and verbascose MRM quantification.

Our results showed that the quantification of the oligosaccharides added in the post-extraction phase was not disturbed by the plant matrix, as reflected in the percentage of oligosaccharide recovered, which ranged from 91.3 to 101.9% (Table 2). These findings therefore confirmed that the legume matrix effect was unlikely be a problem for raffinose, stachyose and verbascose MRM quantification under the conditions optimized in the present study. Similarly, no matrix effect was observed in the quantification of oligosaccharides by other LC-MS methods reported in (Jorge et al., 2017).

Table 3
Raffinose, stachyose, verbascose and total oligosaccharide content (g/100 g) in 28 different legumes.

Legumes			Raffinose	Stachyose	Verbascose	Total oligosaccharides
Genus	*Specie*	*Variety*				
Cicer	*C. arietinum*	"Castellano" chickpea	0.85 ± 0.00*c*	2.59 ± 0.04*i*	0.08 ± 0.00*q*	3.52 ± 0.04*jk*
		"Pedrosillano" chickpea	0.79 ± 0.01*d*	2.58 ± 0.02*i*	0.08 ± 0.04*q*	3.45 ± 0.04*kl*
Glycine	*G. max*	Soybean	0.61 ± 0.04*e*	4.26 ± 0.05*bc*	0.13 ± 0.00*opq*	5.00 ± 0.07*c*
Lens	*L. esculenta*	Beluga black lentil	0.24 ± 0.00*no*	2.08 ± 0.01*j*	0.75 ± 0.01*g*	3.07 ± 0.03*n*
		"Castellana" lentil	0.28 ± 0.01*k*	2.41 ± 0.16*i*	0.77 ± 0.03*fg*	3.46 ± 0.19*kl*
		French lentil	0.22 ± 0.01*op*	2.51 ± 0.08*i*	0.99 ± 0.03*e*	3.71 ± 0.12*ij*
		"Pale Pardina" lentil	0.25 ± 0.01*lmn*	2.21 ± 0.10*j*	0.81 ± 0.03*f*	3.28 ± 0.14*lm*
		"Pardina" lentil	0.27 ± 0.00*klm*	2.55 ± 0.03*i*	0.51 ± 0.01*h*	3.34 ± 0.03*klm*
		Red lentil	0.24 ± 0.01*mno*	2.42 ± 0.11*i*	0.55 ± 0.04*h*	3.21 ± 0.14*mn*
Lupinus	*L. sp.*	Lupin	1.23 ± 0.03*a*	4.19 ± 0.08*c*	1.54 ± 0.02*d*	6.95 ± 0.08*b*
Phaseolus	*P. coccineus*	"Judión de la Granja" bean	0.33 ± 0.01*j*	4.42 ± 0.19*b*	0.21 ± 0.01*klm*	4.96 ± 0.21*c*
	P. vulgaris	"Arrocina" vean	0.54 ± 0.02*f*	4.20 ± 0.27*c*	0.12 ± 0.00*pq*	4.86 ± 0.27*c*
		Black vean	0.18 ± 0.00*r*	3.37 ± 0.16*g*	0.26 ± 0.01*jk*	3.81 ± 0.18*hi*
		"Caparrón" vean	0.22 ± 0.01*op*	3.71 ± 0.13*e*	0.29 ± 0.01*j*	4.22 ± 0.15*ef*
		Cinnamon vean	0.17 ± 0.01*r*	4.15 ± 0.12*c*	0.17 ± 0.01*mnop*	4.49 ± 0.13*d*
		Fageolet (green) vean	0.46 ± 0.01*h*	3.08 ± 0.05*h*	0.17 ± 0.00*mnop*	3.71 ± 0.04*ij*
		"Granja Asturiana" bean	0.34 ± 0.02*j*	3.44 ± 0.05*g*	0.22 ± 0.00*klm*	4.00 ± 0.07*gh*
		Harricot vean	0.22 ± 0.01*op*	3.72 ± 0.08*e*	0.19 ± 0.00*lmn*	4.12 ± 0.09*efg*
		Kidney vean	0.21 ± 0.00*pq*	3.37 ± 0.12*g*	0.14 ± 0.00*nop*	3.72 ± 0.13*ij*
		Pinto vean	0.13 ± 0.00*s*	3.65 ± 0.14*ef*	0.26 ± 0.02*jk*	4.04 ± 0.15*fg*
		Red vean	0.19 ± 0.00*qr*	3.82 ± 0.08*de*	0.24 ± 0.01*jkl*	4.26 ± 0.07*e*
		Virgin vean	0.22 ± 0.00*op*	3.69 ± 0.06*ef*	0.27 ± 0.00*jk*	4.18 ± 0.06*efg*
Pisum	*P. sativum*	Green pea	1.02 ± 0.04*b*	3.67 ± 0.08*ef*	4.52 ± 0.08*a*	9.22 ± 0.16*a*
Vicia	*V. faba*	Broad vean	0.39 ± 0.00*i*	1.45 ± 0.04*k*	3.21 ± 0.09*b*	5.04 ± 0.13*c*
Vigna	*V. angularis*	Adzuki vean	0.28 ± 0.01*kl*	3.52 ± 0.14*fg*	0.36 ± 0.07*i*	4.15 ± 0.14*efg*
	V. mungo	Urd vean	0.50 ± 0.03*g*	3.96 ± 0.05*d*	0.18 ± 0.00*mno*	4.64 ± 0.06*d*
	V. radiata	Mung vean	0.39 ± 0.01*i*	1.46 ± 0.03*k*	2.66 ± 0.08*c*	4.50 ± 0.12*d*
	V. unguiculata	Black-eyed pea	0.40 ± 0.02*i*	5.76 ± 0.16*a*	0.74 ± 0.01*g*	6.89 ± 0.18*b*
		SS p-value Variety	0.0000	0.0000	0.0000	0.0000
		SS p-value Replicate	0.9098	0.3822	0.6158	0.5520

Values are expressed as mean ± SD (n = 3). Different letters indicate significant differences between varieties. SS refer to sum of squares.

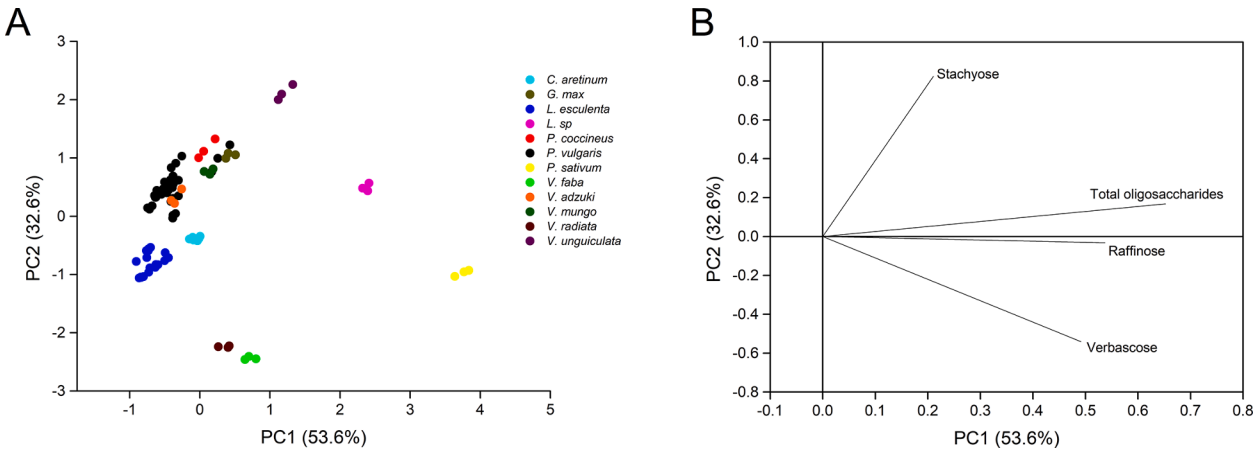

Fig. 3. Principal Component Analysis, score (A) and loading (B) plot of legume samples based on correlation matrix (n = 3). For full name of the legume specie, see Table 3.

3.2.5. MRM quantification of oligosaccharides from legumes

Oligosaccharides extracted from 28 varieties of legumes were analyzed using the optimized procedure, in order to validate the applicability of the MRM method. All the legume samples showed the presence of raffinose, stachyose and verbascose. The oligosaccharide contents in the assessed legumes are summarized in Table 3. Total oligosaccharide content was calculated as the sum of raffinose, stachyose and verbascose and varied in the range 3.07–9.22 g/100 g for beluga lentil and green pea, respectively. Raffinose (g/100 g) ranged from 0.13 (pinto beans) to 1.23 (lupin beans). Stachyose (g/100 g) ranged from 1.45 (broad beans) to 5.76 (black-eyed peas). Verbascose (g/100 g) ranged from 0.08 (chickpeas) to 4.52 (green peas).

A Principal Component Analysis (PCA) was performed, in order to explore whether the oligosaccharides in legumes differ according to the species evaluated (Fig. 3). The two first principal components (PC1 and PC2) accounted for 86% of the total variance (PC1: 53.6%; PC2: 32.6%), with three samples standing out with higher positive values over the PC1. In fact, the total oligosaccharide content was similar in most species (3–5 g/100 g, where *Lens esculenta* and *Cicer arietinum* showed the lowest values – 3.34 and 3.48 g/100 g, respectively), with the exception of *Pisum sativum*, *Lupinus* sp. and *Vigna unguiculata,* which presented higher contents (9.22, 6.95 and 6.89 g/100 g, respectively) (Table 3).

Interestingly, the legume species had an impact on the groups of samples observed. Although the content of oligosaccharides was similar in most of the species, as previously mentioned, the results showed that the ratio of oligosaccharide (raffinose, stachyose and verbascose) content to total oligosaccharides had species-specific differences, as different varieties from the same species had very similar distribution modes.

For most of legume species, stachyose was the predominant (60–92%), except for *Vicia faba*, *Vigna radiata* and *Pisum sativum* which presented higher relative verbascose contents (64, 59 and 49%, respectively). All varieties of *Lens esculenta* had a higher relative content of verbascose than raffinose (6–8%, 67–77% and 15–27%, for raffinose, stachyose and verbascose, respectively), whereas *Cicer arietinum* varieties presented higher relative contents of raffinose than verbascose (23–24%, 74–75% and 2%, for raffinose, stachyose and verbascose, respectively). In contrast, similar relative contents of raffinose and verbascose were found for the varieties of *Phaseolus vulgaris*, which stood out for the highest relative contents of stachyose (3–12%, 83–92% and 2–7%, for raffinose, stachyose and verbascose, respectively). Near the *Phaseolus vulgaris* group were the varieties from the species *Phaseolus coccineus* (7%, 89% and 4%, for raffinose, stachyose and verbascose, respectively), *Vigna angularis* (7%, 85% and 9%, for raffinose, stachyose and verbascose, respectively), *Vigna mungo* (11%, 85% and 4%, for raffinose, stachyose and verbascose, respectively) and *Glicine* max (12%, 85% and 3%, for raffinose, stachyose and verbascose, respectively), which presented similar oligosaccharide distributions. Although *Vigna unguiculata* also showed a similar distribution pattern (6%, 84% and 11%, for raffinose, stachyose and verbascose, respectively), its higher total oligosaccharide content moved it toward higher positive values over the PC1.

Differences in the oligosaccharide content of various legumes have previously been described in the literature (Brummer, Kaviani, & Tosh, 2015; Fan et al., 2015; Han & Baik, 2006; Tosh et al., 2013; Wang, Hatcher, Tyler, Toews, & Gawalko, 2010; Xiaoli et al., 2008). However, to the best of our knowledge, our research involved the most extensive sample collection of legumes (28 different varieties, 12 different species, 8 different genera). Although, in general, our results fall within the range reported for individual and total oligosaccharide content, some authors found non-detectable amounts of verbascose in legumes such as chickpeas and soybeans (Han & Baik, 2006). This could be related to the fact that the occurrence of individual α-galactosides seems to be genetically influenced and dependent on the environment (Trugo, Almeida, & Gross, 1988). In addition, a lower sensitivity of the detectors used for the analyses could be behind such observations. Fan et al. (2015) also observed that the distribution modes of non-digestible oligosaccharides in different varieties of the same species had strong similarity, especially between *Glycine* and *Phaseolus*, as reported in the present study. These authors also highlighted mung beans and broad beans where the relative content of verbascose predominates, which is in keeping with our findings.

All the oligosaccharide determined with the method developed in the present study showed significant differences among legume varieties, whereas no significant differences were observed between replications (Table 3), further emphasizing the reproducibility of the proposed optimized method.

4. Conclusions

The method described in this study can be used to separate raffinose, stachyose and verbascose in a 10-minute run and has been validated over a broad concentration range, providing good linearity, accuracy, intra- and inter-day precision, and approaching one of the highest sensitivities reported so far. This research has demonstrated the effect of both the extraction solvent and the number of extraction cycles on the extractability of oligosaccharides from legumes, proposing a short, low-cost, eco-friendly procedure with a high recovery rate extraction, good repeatability and reproducibility, while no plant-matrix effects were demonstrated. Finally, the method applied to the screening of 28 different legumes revealed species-related traits for the oligosaccharide distribution. For most legume species, stachyose predominated and

Phaseolus vulgaris recorded the highest relative content of stachyose. The lowest total oligosaccharide content was found within the *Lens esculenta* (beluga lentil) species, whereas *Pisum sativum* stood out as the richest source of these prebiotics, thus suitable as a functional food ingredient.

A validated simple, fast and reliable analytical tool has therefore been presented for oligosaccharide quantification and its successful application to a legume food matrix. Furthermore, the largest collection of legumes screened for their oligosaccharide content has been presented, providing useful information for food processors when designing functional foods.

CRediT authorship contribution statement

Virginia Prieto-Santiago: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **María del Mar Cavia:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Francisco J. Barba:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Sara R. Alonso-Torre:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Celia Carrillo:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130761.

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