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Stability study and validation of a liquid chromatography tandem mass spectrometry method for the quantitative analysis of polyphenols in fish feed ingredients

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A B S T R A C T

The perception of polyphenols as a safe, healthy, and sustainable solution for replacing synthetic antioxidants has been an important factor for their rapid growing in the global food market. Therefore, it is essential to use reliable methods for their quantification in commercial products intended for animal or human consumption. The purpose of this study is to evaluate the performance of some solvents used for the extraction of selected polyphenols, explore their stability under different experimental conditions, and validate a liquid chromatography tandem mass-spectrometry method for their quantification in commercial fish feed ingredients by using the standard addition method. The regression models for gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid were linear in the range 0–30 μg/mL, limit of detection and quantification around 0.03 and 0.1 μg/mL, respectively, and accuracy within ± 15 % of the nominal concentrations. The method was successfully applied to the determination of specific polyphenols in commercial fish feed ingredients supplemented with polyphenols from olive and rosemary extracts.

1. Introduction

Natural antioxidants are naturally occurring compounds that possess antimicrobial activity and can decrease oxidation processes in different systems (e.g., human, animal, food) [1,2]. These specific properties are of interest for the food industry, where synthetic antioxidants, such as butylhydroxyanisol (BHA), butylhydroxytoluene (BHT), or ethoxyquin are generally used as preservatives to increase the storage life of food products. Unfortunately, in recent years synthetic antioxidants have attracted negative attention due to the risk they pose to animal and human health [3,4], which has ultimately led to the withdrawal of some of them from the market. For instance, in 2017 the European Union Commission banned the use of ethoxyquin due to its harmful effects on humans, animals and the environment. The United Nations Environment Programme and the Food and Drug Administration have flagged BHT and BHA as potentially harmful synthetic chemicals based on health and environmental hazards considerations [4–7]. BHA interferes with normal hormone function [8], inhibits cell–cell communication [9] and has been labelled as a potential human carcinogen [10,11]. BHT has been associated with an increased incidence of liver tumours in laboratory rodents [12] and with a moderate to high potential for bioaccumulation in aquatic species [5].

The negative aspects of synthetic antioxidants emphasize the importance of searching for natural alternatives, such as polyphenols that are a type of phytochemical compounds, widely distributed in herbs, spices, seeds, fruits, vegetables, olive oil and algae. Polyphenols are perceived by the food industry as safe and healthy substitutes for synthetic antioxidants to extend shelf life and inactive pathogens [13]. Their use as ingredients, additives or supplements in the animal and human food industry is becoming increasingly popular due to their impact on oxidative stress which in the meat and dairy product sectors is reflected in the lipoperoxidation of the final product [1].

The potential health benefits of polyphenols are considered the major force driving their growth in the global food market [14]. It is

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forecasted that the rising adoption of supplementing food products with polyphenols by manufacturers will propel the market forward causing the fastest compound annual growth rate of 7.1 % over the period of 2024–2031 [15]. In addition, the global interest in polyphenols has cause an increase in plant-based research that will continue to expand, especially for their potential to overcome the human/animal health issues ascribed to ethoxyquin, BHT and BHA [4]. In this sense, the research on polyphenols is predominantly conducted by investigating the physiological effects of specific plant materials on living organism [16], and by identifying qualitatively all the polyphenols present in the plant material by nuclear magnetic resonance [17], mass spectrometry [18] and near infrared [19].

The physiological impact of plant-based polyphenols on living systems should be based on quantitative levels of specific polyphenols rather than total profiles. Unfortunately, the quantification of specific polyphenols remains a challenging area that should be promoted, especially in the analysis of food products intended for human and animal consumption and whose compositional labels are lacking information about the content of specific polyphenols.

It is anticipated that the quantification of plant-based polyphenols is a rather difficult task due to the relatively small stock of commercially available standards compared with current myriad of qualitatively characterized polyphenols that might be one of the factors behind the lack of appropriate quantitative documentation. However, the importance of validating quantitative methods is evidenced by dietary studies reporting detrimental effects of polyphenols in a dose-dependent manner [16,20]. For instance, common carp fed diets supplemented with peel pomegranate, rich in gallic acid, revealed similar growth performance at 0.0 and 0.93 g/kg, and significant growth deterioration at 1.85, 2.78 and 3.71 g/kg [16]. Similarly, Chinese seabass fed diets supplemented with grape seeds (rich in flavanol) at 0, 1, and 2 g/kg exhibited reduced feed intake, intestinal injury, metabolic disorder, and depressed growth at 2 g/kg of polyphenol polymers [20]. Adverse outcomes have also been documented from polyphenolic botanical extracts in beverages intended for human consumption [21].

The previous observations highlight the importance of validating sensitive and selective analytical methods for the determination of specific polyphenols in commercial fish feed ingredients fortified with different plants. Liquid chromatography mass spectrometry (LCMS) with electro spray ionization (ESI) is a popular method to determine (from few to over hundreds) polyphenol compounds in a wide variety of samples, such as olive products [22,23], plants and its extracts [24–30], craft beers [31], and wine [32]. The major flaws in the proposed LCMS strategies have been the quantification of the samples based on external standard calibrations without considering the potential effect of the matrix on the polyphenol signals or the use of structurally inappropriate internal standards, as indicated in Table 1.

Although adverse outcomes on fish have been documented after using fish feed supplemented with polyphenols from plant material [16,20], the global market for fish feed ingredients supplemented with polyphenols carry on growing continuously. Hence, the analysis of commercial fish feed ingredients fortified with a combination of different plant extracts should be evaluated in terms of the most abundant polyphenols present in the specific plant sources. The present research is the first report on the validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of specific polyphenols in commercial fish feed ingredients supplemented with polyphenols from olive and rosemary extracts, with emphasis on compound stability and matrix effects.

2. Materials and methods

2.1. Reagents

Catechin hydrate (>98 %), 3-hydroxytyrosol (>98 %), oleuropein, carnosol, carnosic acid, and gallic acid certified reference materials (TraceCERT ®, CRM) were purchased from Merck Life Science AS (Oslo, Norway). Acetonitrile (99.8 %), acetone (99 %) and formic acid (98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol Optima LCMS grade and ethanol HPLC grade were purchased from Fisher Chemical (Oslo, Norway). Ammonium acetate (reagent grade, ≥98 %), copper (II) chloride dihydrate (≥99.99), neocuproine hydrochloride monohydrate and Trolox were obtained from Merck (Darmstadt, Germany). A Millipore Milli-Q system was used to produce ultrapure water 18 MΩ (Millipore, Milford, CT, USA). Two commercial samples of antioxidant ingredients for fish feed containing a mixture of rosemary (rich in carnosic acid and carnosol) and olive (rich in oleuropein) extracts were supplied by Arctic Feed Ingredients (AFI, Brønnøysund, Norway). The samples were designated as S1 and S2, and only the former was provided with a document stating its content of hydroxytyrosol (13211 mg/kg), carnosol (4012 mg/kg) and carnosic acid (3196 mg/kg).

2.2. Solutions for cupric ion reducing antioxidant capacity (CUPRAC)

Aqueous solutions of $CuCl_2 \cdot 2H_2O$ (10 mM), neocuproine solution (7.5 mM) and ammonium acetate buffer (1.0 M; pH 7) and ethanolic solutions of Trolox (125 μM) and sample (12 mg/mL) were prepared separately, vortex-mixed for 20 s (VWR International, Darmstadt, Germany), centrifuged for 5 min at 1811g (Eppendorf, centrifuge 5810R, Burladingen, Germany) at 20 °C and the supernatant was collected and transferred to new vials.

Table 1
Major flaws in the proposed LCMS strategies for quantifying polyphenols in different samples.

Sample	Number of polyphenols	Matrix effect evaluation	Calibration type	Flaws	Reference
Olives	17	Yes	IS	The Internal standard was physical/chemical different from the analytes	[22]
	40	No	ES		[23]
Plants	48	No	IS	As above [22]	[24]
	35	Yes	SA	A single point was used to evaluate the matrix effect	[25]
	14	Yes	ES	As above [25]	[26]
	18	No	ES		[27]
	108	No	Unspecified		[28]
	15	Yes	IS	The internal standards were appropriate. However, some analytical inconsistencies were detected. For instance, 93 % of the compounds exhibited limit off quantification higher than the lower concentration in the analytical range	[29]
	19	Yes	ES	A single point was used to evaluate the matrix effect. In addition, a matrix effect was observed for a compound without information on how to reduce the matrix influence	[30]
Beers	20	Yes	ES	As above [25]	[31]
Wine	12	Yes	SA	Significant matrix effect in a synthetic matrix but not information of the effect in a real matrix	[32]

IS: Internal Standard; ES: External Standard; SA: Standard Addition

2.3. CUPRAC procedure

The CUPRAC reagent preparation has been reported previously [33]. Briefly, 50 μL of the solutions $CuCl_2 \cdot 2H_2O$, neocuproine, and ammonium buffer solutions were added together into a 96-well microplate and incubated at 37 °C for 15 min. After the incubation 100 μL of Trolox or the sample solution were added to each well, gently stirred and submitted to analysis by measuring the absorbance at 450 nm every minute for 30 min. A reagent blank was produced by adding 100 μL ethanol instead of Trolox solution. The calibration curve is constructed by preparing 5 different concentrations of Trolox between 10 and 50 μM, and the Trolox equivalent antioxidant capacity is estimated by the expression reported elsewhere [34]:

$$\mathrm{TEAC} = \frac{\Delta A \times V_f \times V_i}{\varepsilon \times V_s \times W_s \times h \times 1000}$$

where the terms represent the Trolox equivalent antioxidant capacity (TEAC mol/g), the difference in absorbance between sample and blank (ΔA), the final volume of the assay solution per well ($Vf = 0.25$ mL), the initial volume of the sample ($Vi = 25$ mL), the Trolox molar absorptivity in the CUPRAC reagent ($\varepsilon = 3.07 \times 10^{-2}$ L $\times$ mol^{-1} $\times$ cm^{-1}), the volume of the sample used in the assay ($Vs = 0.100$ mL), the weight of the sample ($W_s = 0.3$ g) and the height of the solution in the well ($h = 0.5$ cm).

2.4. Sample extraction

Methanol was the solvent used in the extraction and its selection was based on preliminary extraction experiments where other solvents and conditions were tested. The fine dry powdery sample was weighted (~300 mg) and dissolved in 25 mL of methanol, magnetically stirred for 25 min, further diluted with 15 mL of methanol, centrifugated at 1620 × g for 5 min (Eppendorf AG centrifuge, Hamburg, Germany), and filtered using a Durapore membrane filter, 0.45 μm (Merck, Darmstadt, Germany).

2.5. 2.5. Stability study

A factorial design 3 × 3 × 2 × 2 (supplementary material SM1) was used to evaluate the effect of the storage time (1, 6, and 9 days), temperature (4, −20, and −80 °C), vial color (transparent, amber), and concentration (0.1 and 30 μg/mL) on the stability of the different polyphenols. Two standard solutions containing a mixture of the six polyphenols (gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid) in methanol were prepared at the concentrations indicated by the factorial design and monitoring their chromatographic signals. A total of 36 measurements plus the two initial measurements at 0-day were recorded per polyphenol in triplicate.

2.6. Analytical validation

The LC-MS/MS method was validated for gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid in terms of the performance parameters suggested by the International Committee on Harmonization guidelines [35], more specifically: selectivity, matrix effect, linear range, limit of detection (LOD), limit of quantification (LOQ) and accuracy. All the validation experiments were conducted in triplicate.

The selectivity was evaluated by spiking the samples with equal concentrations of the six polyphenols and evaluating their corresponding extracted ion chromatograms to determine whether the various endogenous components in the sample overlap the signals of the analytical polyphenols.

The matrix effect was evaluated by comparing the sensitivity (or slope) of the external standard curve against post-extraction spiked sample curve (aka standard addition calibration) for each polyphenol. A stock solution containing equal concentrations of the six polyphenols was used to construct the two calibrations, and a concentration range between 0 and 30 μg/mL was prepared to determine the slopes. Suppression or enhancement in sensitivity around ± 20 % was deemed as a non-significant matrix effect [35].

The linear range was evaluated by computing the ratio between the lack-of-fit variance and pure error variance, and also the coefficient of regression of the calibration curves, as suggested by the Analytical Method Committee [36].

The ratio between the standard deviation (σ) and the slope (φ) of the standard addition curve of each polyphenol was used to determine the LOD ($3.3 \times \sigma/\varphi$) and LOQ ($10 \times \sigma/\varphi$) as described elsewhere [37].

The accuracy was estimated by back-calculating the concentrations (C_{bc}) from the standard addition curves and considered acceptable when its value was within ± 15 % of the nominal concentration ($C_{nominal}$) by using the expression:

$$\mathrm{Accuracy}(\%) = 100 \times \left[\frac{C_{bc} - C_{nominal}}{C_{nominal}}\right]$$

2.7. Molecular absorption spectrophotometry

A Victor-X5 2030 multiplate reader spectrometer (PerkinElmer, Turku, Finland) was used to measure the antioxidant capacity of the samples. Absorbance at 450 nm was measured every minute for 30 min, keeping the temperature at 20 °C. The final value after 30 min was used for determining antioxidant capacity.

2.8. Liquid chromatography mass spectrometry

An Agilent ultra-high performance liquid chromatography (UHPLC), coupled to a 6495 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface and iFunnel ionization, was used to quantify the various polyphenols. The UHPLC was equipped with a Zorbax RRHD Eclipse Plus C18, 95 Å, 2.1 × 50 mm, 1.8 μm chromatographic column. The mobile phase delivered at 0.3 μL/min in gradient mode consisted of ultra-pure water with 0.1 % formic acid (solution A) and an equal-volume mixture of acetonitrile and methanol with 0.1 % formic acid (solution B). The flow rate was 0.3 μL/min, the column compartment was held at 30 °C and the injection volume 1 μL. The gradient program was as follows: 0 min 5 % B; 3.5 min 30 % B; 4.0 min 60 % B; 5.5 min 95 % B; 7.5 min 95 % B; 9.5 min 5 % B, 11 min 5 % B. The autosampler was kept at 4 °C and carryover was prevented by consecutive injections of methanol between samples.

The ESI source was used in negative mode at 120 °C, gas flow 19 L/min, nebulizer 20 psi, sheath gas temperature 300 °C, sheath gas flow 10 L/min, capillary voltage 4300 negative mode. The iFunnel parameters were high-pressure RF 150 V and low-pressure RF 60 V. Dwell time was 200 ms. Multiple reaction monitoring (MRM) acquisition was used for the identification and quantification of the compounds. Parameters in the collision cell were optimized for each compound for which an analytical standard was available. The monitored transitions in ion counts units were: m/z 153 → 123 for hydroxytyrosol; m/z 169 → 125 for gallic acid; m/z 289 → 245 for catechin; m/z 329 → 285 for carnosol; m/z 331 → 287 for carnosic acid and m/z 539 → 307, 275 for oleuropein. The integration of the chromatograms was performed using the MassHunter Qualitative Navigator software (version 10.0).

2.9. Statistics

Dunnett's test was used for comparing the chromatographic signals of the selected polyphenols at 0-day against those recorded at the experimental conditions indicated by the factorial design 3 × 3 × 2 × 2. The linearity was evaluated by weighted ($1/\sigma^2$) linear regression

analysis using an automatic Excel routine that allows visualizing the ordinary and weighted calibrations with their corresponding analytical characteristics and residual errors.

3. Results and discussion

3.1. Evaluation of the extraction solvent

Methanol, water, acetonitrile with 0.1 % formic acid, and acetone were evaluated individually by dissolving 300 mg of sample in 25 mL of solvent, using a magnetic stirrer for 25 and 40 min, diluting with 15 mL of solvent, centrifugating, filtering and submitting the filtrated solution to LC-MS/MS analysis. Methanol and water, at the two stirring times, gave similar performance. However, the signals in methanol were slightly better compared to water. Acetonitrile and acetone yielded signals that were 95 and 60 % lower than methanol, respectively, after 25 and 40 min of stirring. Based on the previous observations, methanol was selected as the extraction solvent for further studies. An ultrasonic bath was used for 15 min, as an alternative to reduce the extraction time. However, it was discarded due to the observed increase in the temperature of methanol and high variability in the polyphenol signals. Similar observations have been reported after using an ultrasonic bath and the high variability in the analytical signals ascribed to a degradation process of the compounds [38]. Finally, methanol and 25 min magnetic stirring were chosen as optimal extraction conditions.

3.2. Stability of selected polyphenols

The stability of gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid in terms of the storage time (0, 1, 6, and 9 days), temperature (4, −20, and −80 °C), vial color (transparent, amber), and concentration (0.1 and 30 μg/mL) were evaluated by means of a factorial design. The results for the six polyphenols under the various experimental conditions are presented in Table 2.

Gallic acid: there was a gradual decrease in signal not larger than 10 % up to 6 days for all the tested conditions. However, at day-9 the signals for the low and high concentrations in the amber and transparent vials exhibited a significant reduction over the 30 % at the three tested temperatures.

Hydroxytyrosol: the signals for 0.1 and 30 μg/mL fluctuated within ± 10 % of the initial signal at all the experimental conditions and up to 9 days.

Catechin: the low concentration varied around ± 5 % for all the conditions up to 6 days, however it was dramatically reduced at day-9 in the amber vial at 4 °C (58 %) and −20 °C (55 %), and in in the transparent vial at −20 °C (55 %). The signal for the solution at high concentration decreased gradually around 10 % of the initial signal for all the experimental conditions up to 9 days.

Oleuropein: the variability of the signals under the various experimental conditions varied around ± 10 % respect to the initial signal at 0-day. The only remarkable feature was the non-significant increase in signal (over 10 %) of the 0.1 μg/mL solution in the transparent vial at 4 and −20 °C at day 9.

Table 2
Results of the factorial design used to evaluate the effect of the storage time, temperature, vial color and concentration on the stability of the different polyphenols. The white and brown stripes indicate the transparent and amber vials, respectively. The results are expressed as percentage of variation of a signal relative to the solution at 0-day. Cells without value represent a percentage of variation within ± 10 %.

Concentration (μg/mL)	Storage time (days)	Temperature (°C)	Gallic acid	Hydroxytyrosol	Catechin	Oleuropein	Carnosol	Carnosic acid
0.1	0							
0.1	1	4						
0.1	1	−20						
0.1	1	−80						
0.1	6	4					>30 %	>300 %
0.1	6	−20					>30 %	>300 %
0.1	6	−80					>30 %	>300 %
0.1	9	4	>30 %			>10 %	>30 %	>300 %
0.1	9	−20	>30 %		>50 %	>10 %	>30 %	>300 %
0.1	9	−80	>30 %				>30 %	>300 %
0.1	1	4						
0.1	1	−20						
0.1	1	−80						
0.1	6	4					30 %	>300 %
0.1	6	−20					30 %	>300 %
0.1	6	−80					30 %	>300 %
0.1	9	4	30 %		>50 %		30 %	>300 %
0.1	9	−20	30 %		>50 %		30 %	>300 %
0.1	9	−80	30 %				30 %	>300 %
30	0							
30	1	4						
30	1	−20						
30	1	−80						
30	6	4						
30	6	−20						
30	6	−80						
30	9	4	>30 %					
30	9	−20	>30 %					
30	9	−80	>30 %					
30	1	4						
30	1	−20						
30	1	−80						
30	6	4						
30	6	−20						
30	6	−80						
30	9	4	30 %					
30	9	−20	30 %					
30	9	−80	30 %					

Carnosol: the signals for the low concentration were stable up to 1 day and exhibited a significant increase (over 30 %) at 6 and 9 days for all the conditions. The signals for the high concentration under the different experimental conditions were stable within ± 10 % up to 9 days.

Carnosic acid: it displayed a similar behavior than carnosol for both concentrations in terms of signal stability for the various experimental conditions. However, only the low concentration exhibited an increase in signal over 300 % at day 6 and 9 for the three tested temperatures.

The extraction of the common features for the six polyphenols in Table 2 indicated that the six polyphenols were consistently stable up to 1 day at the different conditions at low concentration (0.1 μg/mL). At a concentration level (30 μg/mL) the six polyphenols were stable up to six days in the different vials (transparent, amber) and at the different tested temperatures (4, −20, and −80 °C).

The samples were extracted by using optimal extraction conditions (methanol and 25 min stirring time) prior to the validation study. Six aliquots (1 mL) of the resulting methanolic solution were equally divided into three transparent and amber vials, stored at −80 °C and monitored for three consecutive days. The results indicated that the chromatographic signals were stable in the considered frame of time. The protection of carnosol by carnosic acid that has been reported in stability studies [39] was neither observed in the standard mixture nor in the real samples (S1 or S2).

3.3. LC-MS/MS validation

The quantitative LC–MS/MS method for gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid was validated with respect to selectivity, linear range, LOD, LOQ and recovery by using freshly prepared solutions.

3.3.1. Selectivity

The evaluation of the extracted ion chromatograms revealed that the LC-MS/MS method can determine gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid without interference from other endogenous components in the sample (Fig. 1).

3.3.2. Matrix effect

The matrix effect is a complex phenomenon that can significantly affect the accuracy and precision of the analytical results, and in the present research was evaluated by comparing the slopes of the external standard and standard addition calibrations for the six polyphenols. The matrix effect was regarded as negligible, under the premise that the numerical ratio between slopes (addition-standard/external-standard) is within the interval 0.9–1.1, that represents a variation within ± 10 % when compared to a theoretical unity ratio. The matrix effect was negligible for catechin, hydroxytyrosol and gallic acid as reflected by relationship between slopes of 1.04, 1.06 and 0.99, respectively. However, significant matrix effects were observed in those polyphenols mostly found in olive and rosemary extracts. For instance, the ratio of the standard addition to external standard slope for oleuropein and carnosol (major compounds in olive and rosemary extract, respectively) showed an enhance in sensitivity of 3.84 and 1.93, respectively. In contrast, the same relationship for carnosic acid (another major compound in rosemary extract) was 0.47, indicating a significant negative impact of the sample matrix on the analytical signal.

These results reflect the importance of understanding in advance the

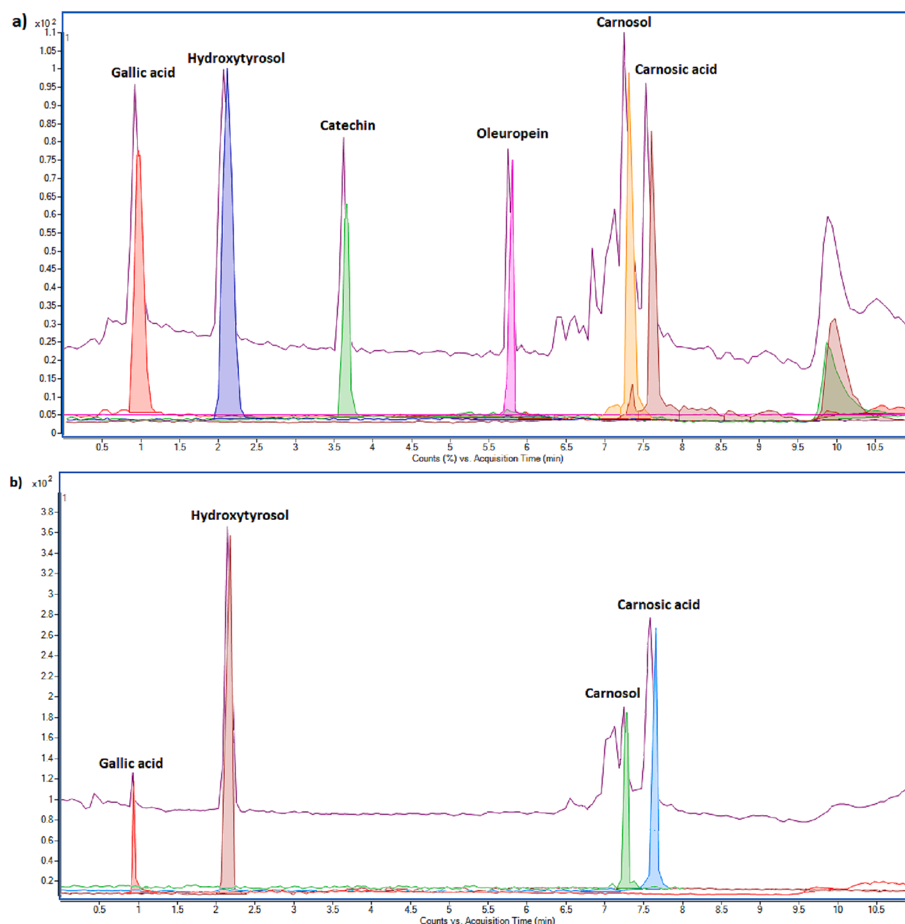

Fig. 1. Total and extracted ion chromatograms to indicate the selectivity of the LC-MS/MS method towards the analyzed polyphenols in commercial fish feed ingredient samples a) S1 and b) S2.

behavior of the different polyphenols in real samples, rather than using external standard calibrations without previous knowledge of the positive/negative impact of the matrix on the analytical signals. The results also prevent using a single internal standard that does not resemble the molecular structures of the target analytes under the belief that it will compensate simultaneously for enhancement/suppression effects. Stable isotopically labeled internal standards (SIL-IS) are recommended for quantitative analysis, however in cases where SIL-IS are not available structural analogues may be used instead. Some researchers have proposed a validated LC-MS/MS using salicylic acid as internal standard to quantify 48 polyphenols in solid residues from the essential oil industry, without studying the matrix effects and unaware of the structural discrepancies between salicylic acid (138 g/mol; $C_8H_{10}O_2$) and the 48 determined polyphenols, including carnosol (330 g/mol; $C_{20}H_{26}O_4$) and carnosic acid (332 g/mol; $C_{20}H_{28}O_4$) [24]. Lack of matrix effects have been reported in the determination of polyphenols in table olives by a validated LC-MS/MS method using hydroxyphenyl-ethanol (137 g/mol; $C_7H_6O_3$) as a single internal standard for 17 polyphenols, including structurally complex molecules, such as oleuropein (541 g/mol; $C_{25}H_{32}O_{13}$) or verbascoside (625 g/mol; $C_{29}H_{36}O_{15}$) [22].

The present study rules out the use of the external standard method as a quantitative strategy for polyphenols in real samples. Also, the lack of appropriate commercial internal standards allows concluding that the standard addition method in conjunction with LC-MS/MS is an appropriate strategy to compensate for the observed enhancement/suppression effects and consequently, it is a suitable approach for the quantification of polyphenols in commercial fish feed ingredients.

3.3.3. Linearity

Considering the observed enhancement/suppression matrix effects in those polyphenols mostly found in olive and rosemary extracts, the linearity for the various polyphenols was assessed by using standard addition calibrations. The linearity was studied by using a 12-level concentration range that revealed a proportional increase in the variance of the chromatographic signals (σ^2_{signal}) over the concentration range for all the polyphenols (data showed as supplementary material SM2). Therefore, weighted regressions were calculated using the inverse of the signal variance ($1/\sigma^2_{signal}$) as weighting factor. The behavior of the residuals after performing ordinary and weighted regression is presented as supplementary material (SM3), and the analytical characteristics of the six mathematical models such as range, weighted equation, statistical Fisher ratio (experimental/tabulated), coefficient of regression (r^2), LOD, LOQ and accuracy are described in Table 3.

It must be emphasized that the literature on method validation for polyphenols regards a value of r^2 close to unity as sufficient evidence to conclude that a calibration function is linear, oblivious to the fact that the regulatory bodies on validation recommend the use of appropriate statistical methods and most important, they do not suggest using the numerical value of r^2 as a sole indicator of linearity. A recent work on the extraction of phenolic compounds from olive leaves using deep eutectic solvents highlighted the importance of the lack of fit test to support mathematical model validity [40], however its reported quantitative analysis was based on external calibration curves without studying the impact of the sample matrix on the analytical determination. In the present research, in addition to the r^2 value, the experimental Fisher test, describing the relationship between the lack-of-fit to pure error variances with 10 and 24 degrees of freedom, respectively, was calculated and compared against its equivalent tabulated value at the 95 % confident level. A statistical Fisher ratio ($F_{experimental}/F_{tabulated}$) lower than 1 along with a r^2 value close to 1 are reliable indicators to establish the linearity of a model. Based on these assumptions the validity of the mathematical models to describe the behavior of the signal as a function of the analytical concentration was positively confirmed for gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid (Table 3).

The six models were used to determine the method accuracy by introducing the experimental signals back into the calibration model to determine the estimated concentration in relation to the nominal value. The accuracy for the six polyphenols was within the ± 15 % interval of variation suggested by the ICH and regarded as acceptable for the determination of polyphenols in fish feed ingredients.

3.4. Antioxidant capacity and quantitative analysis of commercial samples

The antioxidant capacity was measured in triplicate samples of S1 and S2 and the results expressed as Trolox equivalents (TE) were 22.77 ± 4.90 and 36.69 ± 3.41 mol/g, respectively. The validated LC-MS/MS method was used to determine the levels of natural antioxidants (gallic acid, hydroxytyrosol, catechin, oleuropein, carnosol and carnosic acid) in two commercial fish feed ingredients supplemented with plant extracts that were designated as S1 and S2. The presence and content of the various antioxidants varied between the samples (Table 4). Even though ingredients S1 and S2 were characterized by six and four polyphenols, respectively, the antioxidant capacity of the latter was 1.6 times the value of the former. This result indicates the lack of correlation between polyphenol content and antioxidant capacity. A recent study on the

Table 4
Concentrations of polyphenols in commercial fish feed ingredients supplement with olive and rosemary extracts by means of the validated LC-MS/MS method. The values are expressed as average ± standard deviation (n = 3).

	Commercial product S1	S2
TEAC (g/mol)	22.77 ± 4.90	36.69 ± 3.41
	Concentration (mg/kg)	
Gallic acid	0.75 ± 0.02	511 ± 71
Hydroxytyrosol (13211)	12278 ± 247	6807 ± 471
Catechin	1.03 ± 0.02	
Oleuropein	3.62 ± 0.07	
Carnosol (4012)	4935 ± 99	2369 ± 190
Carnosic acid (3196)	3301 ± 66	4496 ± 764

Bracketed numbers are the declared concentrations in mg/kg only for S1

Table 3
Analytical characteristics of the standard addition curves for the fish feed ingredient sample designated as S1. The F-test ratio ($F_{experimental}/F_{tabulated}$) is based on 10 and 24 degrees of freedom for lack-of-fit error and pure error, respectively, at the 95 % confidence level ($F_{tabulated}$ = 2.255). Matrix effect is defined as the ratio between the slopes of the standard addition and external standard calibration curves.

	Retention time (min)	Weighted regression (0–30 μg/mL)	F-test ratio	r^2	LOD (μg/mL)	LOQ (μg/mL)	Accuracy (%) min	Accuracy (%) max	Matrix effect
Gallic acid	0.96	$y = 7.63 \times 10^4 \times [C] + 4.63 \times 10^1$	3.7×10^{-32}	0.998	0.02	0.07	−2.66	4.40	0.99
Hydroxytyrosol	2.11	$y = 1.07 \times 10^5 \times [C] + 1.06 \times 10^6$	1.2×10^{-14}	0.998	0.02	0.07	−12.51	8.82	1.06
Catechin	3.66	$y = 2.19 \times 10^4 \times [C] + 1.83 \times 10^1$	3.2×10^{-32}	0.997	0.03	0.09	−3.69	5.35	1.04
Oleuropein	5.80	$y = 3.35 \times 10^4 \times [C] + 9.8 \times 10^1$	9.7×10^{-32}	0.998	0.02	0.07	−3.00	4.00	3.84
Carnosol	7.33	$y = 9.33 \times 10^4 \times [C] + 3.72 \times 10^5$	6.3×10^{-15}	0.997	0.03	0.09	−8.45	14.98	1.93
Carnosic acid	7.56	$y = 5.47 \times 10^4 \times [C] + 1.46 \times 10^5$	6.7×10^{-14}	0.997	0.03	0.09	−13.31	7.91	0.47

y = signal in ion counts; [C] = concentration in μg/mL.

antioxidant capacity of 20 different samples of extra virgin olive oil (EVOO), has found that the total polyphenol content in a sample designated as EVOO-4 was three times higher than sample EVOO-9, however its antioxidant capacity was three times lower than EVOO-9 [41].

The accuracy for sample S1 was determined by the degree of closeness between experimental and declared concentrations for hydroxytyrosol, carnosol and carnosic acid. It is important to mention that the declared concentrations of polyphenols in this sample are based on experimental values provided by the manufacturer of the olive and rosemary extracts. The accuracy for hydroxytyrosol (-7.06 %) and carnosic acid (3.27 %) indicated that their concentrations were within ± 15 % of the declared concentrations. However, for carnosol (23.01 %) the result seems to suggest that this commercial product contains more carnosol than expected. Considering the resolution power of the triple quadrupole mass spectrometer, the use of the standard addition method, and the back-calculated accuracy results for the six polyphenols, then it is likely that the found content of carnosol of 4935 ± 99 mg/kg in S1 is the actual concentration in the product rather than the declared value of 4012 mg/kg (Table 4). Although the two products (S1 and S2) have been supplemented with olive and rosemary extracts, the differences between them are notable and may be ascribed to differences in the content of polyphenols in the initial plant extracts. Previous researchers have reported seasonal variations of rosemary and olive extracts [42,43]. In addition, the manufacturer stated that S1 and S2 were prepared in 2022 and 2023, respectively, which supports the hypothesis of differences in composition of the initial raw material.

4. Conclusions

To the authors knowledge, this is the first report on the validation of a LC-MS/MS method for quantifying selected polyphenols in commercial fish feed ingredients supplemented with polyphenols from plant extracts using the standard addition method. The study highlighted the importance of performing an a priori evaluation of the stability and matrix effects to decide the correct calibration strategy.

The present research emphasised that reporting linearity results is an important component in method validation, due to the various parameters that can be estimated from the relationship between concentration and signal (e.g., range, regression equation, r^2, LOD, LOQ, accuracy). The implicit interconnection between these parameters should be preserved, otherwise will affect the reliability of the full report. For instance, a comprehensive study on the LC-MS/MS determination of flavonoids and salicylic acid from willow bark samples using solid phase extraction and various well-selected isotopically labeled internal standards has reported for instance a linear range and LOQ for catechin of 0.0488–100 ng/mL and 0.38 ng/mL, respectively, oblivious to the fact that the LOQ was almost eight times higher than the lower concentration of the linear range (LCR). Such inconsistency was observed for 14 out of the 15 reported polyphenols where the LOQ/LCR ratio ranged from 3 to 132 [29].

CRediT authorship contribution statement

Viviana Sarmiento: Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kristin Hamre:** Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing, Conceptualization. **Aleksander Arnø:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Joshua Dagogo:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Elisabeth Ødegård:** Investigation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Odd Elvebø:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing. **Pedro Araujo:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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