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To cite this version:

Raúl Domínguez-Perles, Ángel Abellán, Daniel León, Federico Ferreres, Alexandre Guy, et al.. Sorting out the phytoprostane and phytofuran profile in vegetable oils. Food Research International, 2018, 107, pp.619-628. 10.1016/j.foodres.2018.03.013 . hal-02612577

HAL Id: hal-02612577

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Sorting out the phytoprostane and phytofuran profile in vegetable oils

Raúl Domínguez-Perles[a], Ángel Abellán[a], Daniel León[a], Federico Ferreres[a], Alexander Guy[b], Camille Oger[b], Jean Marie Galano[b], Thierry Durand[b], Ángel Gil-Izquierdo[a,*]

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ARTICLE INFO

Keywords:
Vegetable oils
α-Linolenic acid
Plant oxylipins
Saponification
UHPLC-ESI-QqQ-MS/MS

ABSTRACT

Phytoprostanes (PhytoPs) and phytofurans (PhytoFs) are prostaglandin-like compounds, contributing to defense signaling and prevention of cellular damage. These plant oxylipins result from autoxidation of α-linolenic acid (ALA) and have been proposed as new bioactive compounds due to their structural analogies with isoprostanes (IsoPs) and prostanoids derived from arachidonic acid in mammals, which have demonstrated diverse biological activities. The present work assesses a wide range of vegetable oils - including extra virgin olive oils (n = 7) and flax, sesame, argan, safflower seed, grapeseed, and palm oils - for their content of PhytoPs and PhytoFs. Flax oil displayed the highest concentrations, being notable the presence of 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP (7.54, 28.09, 28.67, and 19.22 μg mL^{-1}, respectively), which contributed to a total PhytoPs concentration of 119.15 μg mL^{-1}, and of *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (21.46 μg mL^{-1}). Palm and grapeseed oils appeared as the most appropriate negative controls, given the near absence of PhytoPs and PhytoFs (lower than 0.15 μg mL^{-1}). These data inform on the chance to develop nutritional trials using flax and grapeseed oils as food matrices that would provide practical information to design further assays intended to determine the actual bioavailability/bioactivity *in vivo*.

1. Introduction

Phytoprostanes (PhytoPs) are regio- and stereo-isomeric prostaglandin-like compounds found in plants, being products of α-linolenic acid (ALA, C18:3 n−3) autoxidation (Barbosa et al., 2015; Mueller, 2004). To date, two regioisomeric series (16-G_1-PhytoPs and 9-G_1-PhytoPs) have been proposed as precursors of the diverse classes of cyclic PhytoPs (Jahn, Galano, & Durand, 2010). In the last few years, novel non-enzymatic pathways have been described that lead to the synthesis of phytofurans (PhytoFs) - additional oxygenated metabolites of ALA synthesized under higher oxygen partial pressure (Cuyamendous et al., 2015; Fessel, Porter, Moore, & Sheller, 2002) - so named for their homology with the 3-hydroxy-2,5-disubstituted tetrahydrofuran structures occurring in mammals, derived from arachidonic acid (AA) (isofurans), docosahexaenoic acid (neurofurans), and adrenic acid (dihomo-isofurans) (de la Torre et al., 2015; Roberts & Fessel, 2004). Both PhytoPs and PhytoFs are constitutively synthesized in plant cells in response to increased levels of reactive oxygen species (ROS), being part of the molecular tools of higher plants involved in defense signaling and in the prevention of cellular damage caused by redox imbalance (Loeffler et al., 2005).

The demonstration of the biological activities of PhytoPs and PhytoFs has been based on *in vitro* mechanistic studies, whilst addressing the existing gap of information concerning their actual biological activity *in vivo* requires further experimental support. In this sense, *in vivo* assays (regarding not only bioactivity but also bioavailability) would provide valuable data on underexplored issues essential to the understanding of the biological relevance of plant oxylipins (PhytoPs and PhytoFs) in mammals after their dietary intake (Barden et al., 2009; Dupuy et al., 2016). Indeed, the lack of information on the actual concentration reached in the diverse cells and tissues at a systemic level constitutes a major constraint to the development of mechanistic studies into a realistic mode of action, due to the use of improper concentrations exceeding those occurring *in vivo* and chemical forms not matching those present *in vivo* in cells and tissues.

For the development of nutritional trials, vegetable oils merit

Abbreviations: ALA, α-linolenic acid; AA, arachidonic acid; BHT, butylated hydroxytoluene; MRM, multiple reaction monitoring; PCA, principal component analysis; PhytoFs, phytofurans; PhytoPs, phytoprostanes; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SPE, solid-phase extraction; UHPLC-ESI-QqQ-MS/MS, ultra-high performance liquid chromatography coupled to electrospray and triple quadrupole mass spectrometry

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consideration as sources of PhytoPs and PhytoFs given their broad inclusion in the diet in their raw form. In this regard, though a number of scientific articles have addressed the profile and content of PhytoPs in vegetable oils (Collado-González et al., 2015; Collado-González et al., 2015; Karg et al., 2007), the diversity evaluated has not been enough to identify true controls, essential to retrieve consistent results.

The present study profiles and quantifies PhytoPs and PhytoFs in vegetable oils - including extra virgin olive oil (EVOO) from seven distinct varieties of drupes, as well as argan, flax, sesame, grapeseed, safflower, and palm oils. The qualitative and quantitative data were obtained by UHPLC-ESI-QqQ-MS/MS and inform on the best vegetable oil to be included in further bioavailability and clinical trials. The application of biostatistical techniques allowed the determination of both the correlations between individual compounds and the potential of the information obtained for the categorization of the vegetable oils considered.

2. Materials and methods

2.1. Chemical and reagents

The PhytoPs 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP and the PhytoFs *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (Fig. 1) were synthesized according to procedures described in the literature (Cuyamendous et al., 2015; Cuyamendous et al., 2017; El Fangour et al., 2004; El Fangour, Guy, Vidal, Rossi, & Durand, 2005; Guy, Flanagan, Durand, Oger, & Galano, 2015; Oger, Brinkmann, Bouazzaoui, Durand, & Galano, 2008; Pinot et al., 2008). They were provided by the *Institut des Biomolécules Max Mousseron* (IBMM) (Montpellier, France). Hexane and chloroform were obtained from Panreac (Castellar del Vallès, Barcelona, Spain), Bis–Tris (bis(2-hydroxyethyl)amino-tris (hydroxymethyl)methane) was purchased from Sigma–Aldrich (St. Louis, MO, USA), and all LC–MS grade solvents, methanol, and acetonitrile were from J.T. Baker (Phillipsburg, NJ, USA). The Strata solid-phase extraction (SPE) cartridges used (Strata x-AW, 100 mg 3 mL^{-1}) were acquired from Phenomenex (Torrance, CA, USA).

2.2. Vegetable oil samples: preparation and extraction

The samples consisted of the commercial oils: 0.1° extra virgin olive (*Olea europaea* L.) oils made using the drupe varieties 'Arbequina', 'Coupage', 'Koroneiki', 'Cuquillo', 'Hojiblanca', 'Cornicabra', and 'Picual' (Oilmedros S.L., Jumilla, Spain), and bio-organic virgin flax (*Linum usitatissimum* L.), virgin sesame (*Sesamum indicum* L.), and virgin argan (*Argania spinosa* L.) oils (Laboratorios Almond, NaturGreen, Librilla, Spain). In addition, safflower (*Carthamus tinctorious* L.) seed (Nutrition & Santé Ibérica SL, Sant Cugat del Vallès, Spain), grapeseed (*Vitis vinifera* L.) (La compagnie des saveurs, Novers-Sur-Cher, France), and palm (*Elaeis guineensis*) (Gracomsa alimentaria S.A., Valencia, Spain) oils were also assessed for their content of PhytoPs and PhytoFs. The oils were stored at room temperature (RT), protected from light, until extraction and analysis.

The PhytoPs and PhytoFs present in the oils were extracted following the methodology described by Leung, Chen, Zhong, Yu, and Lee (2014) and Yonny et al. (2015), with minor modifications for its

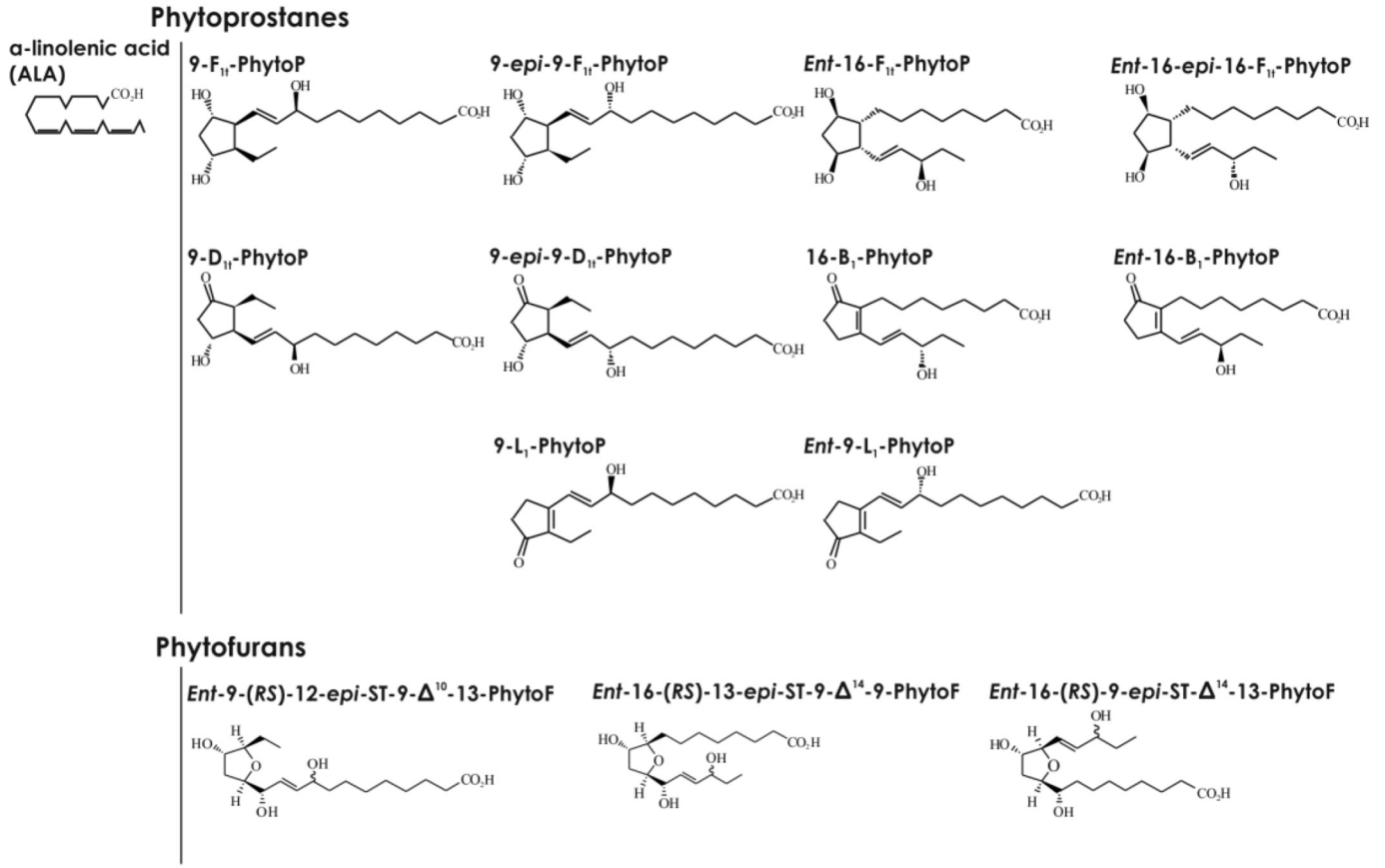


Fig. 1. Chemical structures of the phytoprostanes (PhytoPs) 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, *ent*-16-B_1-PhytoP, 16-B_1-PhytoP, *ent*-9-L_1-PhytoP, and 9-L_1-PhytoP; and the phytofurans (PhytoFs) *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, *ent*-9-(*S*)-12-*epi*-ST-Δ^{10}-13-PhytoF, *ent*-9-(*R*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, assessed in vegetable oils. Names are according to Taber, Morrow, and Roberts 2nd (1997) and Cuyamendous et al. (2016).

adaptation to liquid samples. Briefly, each vegetable oil (1.0 mL) was mixed with 8.0 mL of 0.1% butylated hydroxytoluene (BHT) in methanol (w/v), and the mixture was stirred at 200 *rpm* for 10 min in an orbital shaker. Afterwards, 4.0 mL of chloroform and 1.0 mL of milliQ-water were added and the mixture was stirred at 200 *rpm* for 15 min, at RT, in an orbital shaker before being centrifuged at 1800*g*, for 10 min at 4 °C. The chloroform phase was separated and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry residue was suspended in 2.0 mL of hexane and 2.0 mL of 0.02 M Bis-Tris (pH 7) and kept at 4 °C until further clean-up by SPE.

The SPE clean-up of the extracts was developed using Strata X-AW cartridges

(100 mg 3.0 mL^{-1}), according to the procedure described previously (Collado-González, Medina, et al., 2015; Medina et al., 2012). To reveal the recovery of each analyte, the ratio "(*final concentration − initial concentration*) / *initial concentration*" was determined for three different concentrations within the previously estimated linear range for the separate standards (68.89, 137.78, and 215.28 ng mL^{-1}). The target compounds were eluted with 1.0 mL of methanol and dried using a SpeedVac concentrator. The dry extracts were reconstituted with 200 μL of milliQ-water/MeOH (50:50, v/v), sonicated for 10 min, and filtered through a 0.45-μm filter (Millipore, MA, USA). The recovery was calculated in quintuplicate (n = 5) for each concentration.

2.3. UHPL-ESI-QqQ-MS/MS analysis of phytoprostanes and phytofurans

Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany) according to the methodology described by Collado-González, Medina, et al. (2015), with the inclusion of three additional compounds, which validation is presented in the present work. The analytical column was a BEH C18 (2.1 × 50 mm, 1.7 μm) (Waters, Milford, MA). The column temperatures were both 6 °C (left and right). The mobile phases consisted of milliQ-water/acetic acid (99.99:0.01, v/v) (A) and methanol/acetic acid (99.99:0.01, v/v) (B). The sample injection volume and flow rate were 20 μL and 0.2 mL min^{-1}, respectively. The chromatographic separation was achieved through the following linear gradient (time (min), %B): (0.00, 60.0%); (2.00, 62.0%); (4.00, 62.5%); (8.00, 65.0%); and (8.01, 60.0%). An additional post-run of 1.5 min was used for column equilibration. The MS analysis was conducted by multiple reaction monitoring (MRM) operated in the negative mode, assigning preferential MRM transition to the corresponding analytes (Table 1). The operating conditions regarding the MS parameters were: gas temperature: 325 °C, gas flow: 8 L min^{-1}, nebulizer: 30 psi, sheath gas temperature: 350 °C, jet-stream gas flow: 12 L min^{-1}, capillary voltage: 3000 V, and nozzle voltage: 1750 V. Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies).

2.4. Sensitivity, precision, and accuracy

The sensitivity of the analytical method (referred to the three PhytoFs *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, which were added to the method developed and validated by Collado-González, Medina, et al. (2015)) was evaluated as the limits of detection (LOD) and quantification (LOQ), as the lowest concentration of an analyte that can be detected but not necessarily quantified and quantified, respectively. In our method, the LOD and LOQ were established with an S/N ratio of 3:1 and 10:1, respectively (International Conference on Harmonization (ICH), 1994).

The precision (coefficient of variation (CV%)) and the accuracy (% deviation) of the intra- and inter-day assays were evaluated for three concentrations (three replicates per concentration) of these PhytoFs (68.89, 137.78, and 215.28 ng mL^{-1}) by carrying out two runs per day on five independent days. The concentrations were determined with daily calibration curves. The precision was calculated as "*CV % = (standard deviation / mean)* × 100", whilst the standard deviation informed on the accuracy of the method (FDA, US Department of Health and Human Services, 2001).

2.5. Statistical analyses

All extractions were performed in triplicate (n = 3) and the data were expressed as the means and the least significant differences (LSD). All statistical tests were performed at a 5% significance level using the SPSS 22.0 software package (LEAD Technologies, Inc., Chicago, USA). All the data were subjected to one-way analysis of variance (ANOVA). The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by the Kolmogorov–Smirnov (with Lilliefors correction) and Levene test, respectively. When statistical differences were identified, the variables were compared using Tukey's multiple range test.

Principal component analysis (PCA) was applied as a pattern recognition unsupervised classification method. The number of dimensions kept for data analysis was evaluated by the respective eigenvalues (which should be higher than one), using the total percentage of variance. The number of plotted dimensions was chosen in order to allow meaningful interpretations, thereby ensuring their reliability. Pearson's correlation was conducted and significant correlations were set at $p < 0.05$.

3. Results & discussion

The measurement of PhytoPs and PhytoFs was developed in a suite of vegetable oils, using a newly validated UHPLC-ESI-QqQ-MS/MS method for PhytoFs that is specific, sensitive, reproducible, and robust as well as representing an updated method for PhytoPs, relative to the one previously described by Collado-González, Medina, et al. (2015) that is complemented by including three new analytes (*ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF). Hence, the cross-reactivity between the diverse plant oxylipins is resolved by the method applied in the present work, which provides a clear separation and identification of enantiomers and reduces interferences between compounds. For identification and quantification purposes, two transitions were used for each separate compound: the more intense one for quantification and the additional MRM transition for confirmation of the identity of the compound (Table 1). Indeed, the use of two pairs of MRM transition is recommended by the European Commission (Commission Decision2002/657/EC, 2002) and the International Conference on Harmonization (International Conference on Harmonization (ICH, 1994). These publications noticed the very valuable confirmation of the LC-MS results provided by mass spectral fragmentation and the ability to filter out by mass any impurities interfering in spectrophotometric detectors. In this regard, working in Multiple Reaction Monitoring mode allows to monitor two (or more) transition for each analyte, being used the first one for the quantification of the target compound, whilst the second product ion is monitored for the confirmation of the compound identity. In addition, the compounds under evaluation also showed distinct retention times that guaranteed their identification without any constraint (Table 1).

3.1. Qualitative and quantitative MRM transitions, and analytical selectivity

Because of the occurrence of a free carboxylic group in PhytoPs and PhytoFs, the negative ESI mode gives a more efficient ionization and thus a more proficient detection and quantification of plant oxylipins (Medina et al., 2012). Besides, complementary MRM transitions (qualitative transitions) were included in the method for further confirmation of the identity of the compounds (Table 1). Hence, the analytical

Table 1
UHPLC/MS/MS parameters for the quantification and confirmation of phytoprostanes and phytofurans in vegetable oils.

Compound	Retention time (min)	ESI mode	MRM transition (*m/z*)	Fragmentor (V)	Collision energy (V)
Phytoprostanes					
Ent-16-*epi*-16-F_{1t}-PhytoP[X]	1.583	Negative	327.1 > 283.2[Z]	80	15
		Negative	327.1 > 225.1[Y]	80	15
9-F_{1t}-PhytoP	1.631	Negative	327.2 > 273.1	110	15
		Negative	327.2 > 171.0	110	15
Ent-16-F_{1t}-PhytoP[X]	1.712	Negative	327.2 > 283.2	80	10
		Negative	327.2 > 225.1	80	10
9-*epi*-9-F_{1t}-PhytoP	1.785	Negative	327.2 > 272.8	110	10
		Negative	327.2 > 171.0	110	10
9-D_{1t}-PhytoP	1.791	Negative	325.2 > 307.3	100	4
		Negative	325.2 > 134.7	100	4
9-*epi*-9-D_{1t}-PhytoP	2.022	Negative	325.2 > 307.2	100	7
		Negative	325.2 > 134.9	100	7
16-B_1-PhytoP	2.620	Negative	3.7.2 > 223.2	100	10
		Negative	307.2 > 235.1	100	100
9-L_1-PhytoP	3.079	Negative	307.2 > 185.1	110	7
		Negative	307.2 > 196.7	110	7
Phytofurans					
Ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	0.906	Negative	344.0 > 300.0	110	10
		Negative	344.0 > 255.9	110	10
Ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	1.501	Negative	343.9 > 209.0	90	12
		Negative	343.9 > 201.1	90	12
Ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	1.523	Negative	343.0 > 171.1	90	22
		Negative	343.0 > 97.2	90	22

[Z] Quantification transition.
[Y] Confirmation transition.
[X] Coeluting diastereoisomers quantified together.

selectivity of the method used allowed the differentiation of specific analytes eluting at matching retention times from other matrix components with similar chemical features.

3.2. Calibration curves

The quantification of the PhytoPs and PhytoFs involved the daily preparation of calibration curves using standard solutions. The linearity was assessed using milli-Q-water/methanol (50:50, v/v) solutions of the standards. The calibration curves were fitted using linear regression equations of the type "$y = ax + b$"; the correlation coefficients (R^2) were higher than 0.99 within the concentration range of 10.34 to 344.44 ng mL^{-1}, thus showing the adequate linearity of the analytical procedure. The standard curves were prepared separately for each compound or with a pool of all the standards, with identical satisfactory results.

3.3. Recovery, sensitivity, precision, and accuracy

When samples are cleaned-up by SPE, it is mandatory to evaluate the recovery of the compounds of interest. In addition to the selectivity of the SPE cartridges, the recovery may be affected by several other factors, including chromatography and ionization at the source. In addition, since the matrix-related ion-suppression effect that features in the MS analyses of these compounds, limits dramatically the detection and quantification capacity for some PhytoPs and PhytoFs, recovery tests are mandatory. The Strata X-AW cartridge used for the SPE gave an elevated recovery and good reproducibility (Table 2) for all three PhytoFs (86.5–108.1% for *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; 108.9–121.5% for *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; and 96.8–123.0% for *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF) due to the weak ionic-exchange interaction of its resin.

The LOD and LOQ of the compounds analyzed were, respectively, 4.45 and 14.88 ng mL^{-1} for *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, 2.02 and 6.73 ng mL^{-1} for *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, and 0.10 and 0.34 ng mL^{-1} for *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (Table 3). The intra- and inter-day CV% or precision and the percentage of variation or accuracy were within the acceptable limit (< 15%) set up by the International Conference of Harmonization (ICH, 1994). Actually, the CV % ranged from 2.9 to 13.3% and from 3.1 to 12.7% for the intra- and inter-day multiple determinations, respectively. The accuracies retrieved varied between 85.6 and 103.4% (intra-day) and between 83.0 and 110.5% (inter-day) (Table 4).

Table 2
Recovery of PhytoFs.

Analyte	Added (ng mL^{-1})	Absolute recovery (%)
Ent-9-(RS)-12-epi-ST-Δ^{10}-13-PhytoF	68.89	86.48 ± 4.1
	137.78	108.05 ± 19.4
	215.28	102.71 ± 5.0
Ent-16-(*RS*)-9-epi-ST-Δ^{14}-10-PhytoF	68.89	108.93 ± 22.0
	137.78	121.48 ± 12.1
	215.28	112.66 ± 11.9
Ent-16-(*RS*)-13-epi-ST-Δ^{14}-9-PhytoF	68.89	104.42 ± 18.0
	137.78	96.79 ± 12.1
	215.28	122.96 ± 10.5

3.4. Phytoprostanes and phytofurans profiles in vegetable oils

The assessment of the vegetable oils studied in the present work, regarding their profiles of PhytoPs and PhytoFs, provided accurate information on the compounds present in each matrix and their abundance. The synthesis of PhytoPs and PhytoFs by higher plants is closely dependent on the presence of ALA and molecular oxygen. They are non-enzymatically synthesized and, although these processes do not require metabolic activity of living cells, the formation of ROS in cells (that is related to enzymatic pathways) could play a role in the formation of plant oxylipins (Carrasco-Del Amor et al., 2016; Carrasco-Del Amor et al., 2017; Collado-González et al., 2015; Loeffler et al., 2005). In this regard, since these compounds are end products of the non-enzymatic peroxidation of ALA, their occurrence is strongly conditioned by the features of the processing procedure and the storage conditions, especially in those products obtained from manufacturing processes requiring the breakdown of the food matrix and thus exposure to high

Table 3
Analytical features of the UHPLC-ESI-MS/MS method for the qualitative and quantitative analysis of phytofurans, with indication of the linearity, limit of detection (LOD), and limit of quantification (LOQ) for each compound.

Analyte	Calibration equation	Coefficient of regression	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	y = 101,515.36× + 1733.98	0.9926	4.45	14.88
Ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	y = 54,259.90× + 2055.64	0.9988	2.02	6.73
Ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	y = 73,044.36× + 1974.49	0.9985	0.10	0.34

oxygen pressure (Thoma et al., 2003).

The individual compounds present in the vegetable oils analyzed were identified through the use of their mass spectra - considering their pseudomolecular ion (*m/z*), the most specific fragmentations, and the elution order represented by the retention times (Table 1 and Fig. 1) - according to the method developed by Collado-González, Medina, et al. (2015) and with the use of authentic standards. The number of compounds (PhytoPs and PhytoFs) found decreased as follows: flax oil (11) > safflower oil (7) > EVOO (6) > sesame and argan seed oil (5) > grapeseed and palm oils (4).

These PhytoP and PhytoF profiles should be carefully interpreted since the method applied does not allow the resolution of two mixtures of enantiomers - 16-B_1-PhytoP + *ent*-16-B_1-PhytoP, and 9-L_1-PhytoP + *ent*-9-L_1-PhytoP - which co-elute, displaying also matching fragmentation patterns that necessitate the use of chiral additives and/or columns for their proper resolution Collado-González, Medina, et al. (2015). Hence, in the present work these two pairs of PhytoPs were quantified as 16-B_1-PhytoP and 9-L_1-PhytoP, respectively. Besides, the chromatographic method applied in the present work was not able to separate the two C-16 epimers of the 16-series of F_{1t}-PhytoPs (*ent*-16-*epi*-16-F_{1t}-PhytoP and *ent*-16-F_{1t}-PhytoP).

The PhytoP profile found in the seven classes of EVOO (0.1°) differed from that recorded in olive oils by Collado-González et al., who reported the presence of *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, and 16-B_1-PhytoP Collado-González, Medina, et al. (2015). In the current work their presence in EVOO was not observed, except for 16-B_1-PhytoP, whereas 9-F_{1t}-PhytoP and 9-L_1-PhytoP were found. Diverse factors may be responsible for these apparently contradictory results, particularly the genetic information carried by the separate varieties of olive drupes used to obtain the analyzed oils; this entails distinct concentrations of ALA that could affect the synthesis of PhytoPs and PhytoFs (Imbusch & Mueller, 2000a). The occurrence of individual PhytoPs in the other vegetable oils evaluated was restricted to 9-F_{1t}-PhytoP (argan, sesame, grapeseed, and safflower oils), *ent*-16-*epi*-F_{1t}-PhytoP and *ent*-16- F_{1t}-PhytoP (sesame and safflower oils), 9-*epi*-F_{1t}-PhytoP (argan and grapeseed oils), 9-L_1-PhytoP (argan, sesame, safflower, and palm oils), and 16-B_1-PhytoP (all of them).

With respect to the occurrence of PhytoFs in vegetable oils, to the best of our knowledge, the data available are restricted to flax oil (Cuyamendous et al., 2017). In this regard, determination of the concentrations of PhytoFs in plant foods is essential to complete our knowledge of the occurrence of plant oxylipins; this shows the need for further evaluation of the bioavailability of such compounds. Within the family of PhytoFs, three different regioisomers were analyzed; *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. All three were observed in the seven varieties of EVOO and in flax oil. In contrast, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-9-PhytoF was present in argan, sesame, grapeseed, safflower, and palm oils, whilst *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF was only detected in grapeseed and palm oils and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in none of these vegetable oils. Again, as for the PhytoPs, the diversity of the PhytoFs profiles recorded in the array of vegetable oils assessed seems to be closely related to differences in the genetic backgrounds, as well as to specific features of the production process (Karg et al., 2007; Collado-González, Medina, et al., 2015.

The wide range of PhytoPs and PhytoFs identified in these vegetable oils reinforces the value of this food matrix for testing their bioavailability and biological activity *in vivo*, to demonstrate the potential bioactivity of this family of compounds, mainly supported so far by structural analogies with eicosanoids to which have been attributed several powerful biological actions (Barden et al., 2011). This could be demonstrated by the provision of complementary information on their occurrence *in vivo* after the oral intake of dietary sources and their capacity to modulate the level of expression of biological markers associated with pathophysiological situations in *in vitro* mechanistic studies. In this regard, given these structural coincidences, it has been suggested that PhytoPs and PhytoFs could develop their biological activity through equal molecular pathways, whilst the chemical diversity of the individual compounds could allow variable mechanisms of action and biological properties.

3.5. Quantitative occurrence of phytoprostanes and phytofurans in vegetable oils

Given the high concentration of ALA in vegetable oils used around the world, at the domestic level or in industrial processes (mainly in the baking industry), consumers ingest significant amounts of PhytoPs and PhytoFs, which reach the intestinal tract and are susceptible to absorption and spread at the systemic level (Karg et al., 2007), where they could develop their biological activity. However, to date, there is limited information on the distribution or accumulation of PhytoPs and

Table 4
Intra-day and inter-day coefficients of variation for the determination of PhytoFs.

Analyte	Nominal conc. (ng mL^{-1})	Intra-day			Inter-day		
		Measured	RSD (%)	Accuracy (%)	Measured	RSD (%)	Accuracy (%)
Ent-9-(RS)-12-epi-ST-Δ^{10}-13-PhytoF	68.89	24.72 ± 1.53	13.3	85.6	23.98 ± 2.10	11.2	83.0
	137.78	125.65 ± 2.07	10.7	91.2	124.89 ± 2.10	8.9	90.6
	275.55	264.35 ± 23.83	6.0	95.9	264.12 ± 19.28	7.2	96.1
Ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	68.89	26.12 ± 1.07	10.1	90.4	25.96 ± 0.49	12.7	89.9
	137.78	142.45 ± 1.94	7.7	103.4	144.12 ± 2.92	7.7	104.6
	275.55	279.65 ± 12.97	10.6	101.5	289.89 ± 15.00	11.8	105.2
Ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	68.89	27.89 ± 0.11	5.4	96.5	27.56 ± 0.25	7.4	95.4
	137.78	140.75 ± 8.94	2.9	102.2	152.21 ± 2.17	3.1	110.5
	275.55	283.98 ± 6.67	4.0	103.1	292.65 ± 6.82	4.9	106.2

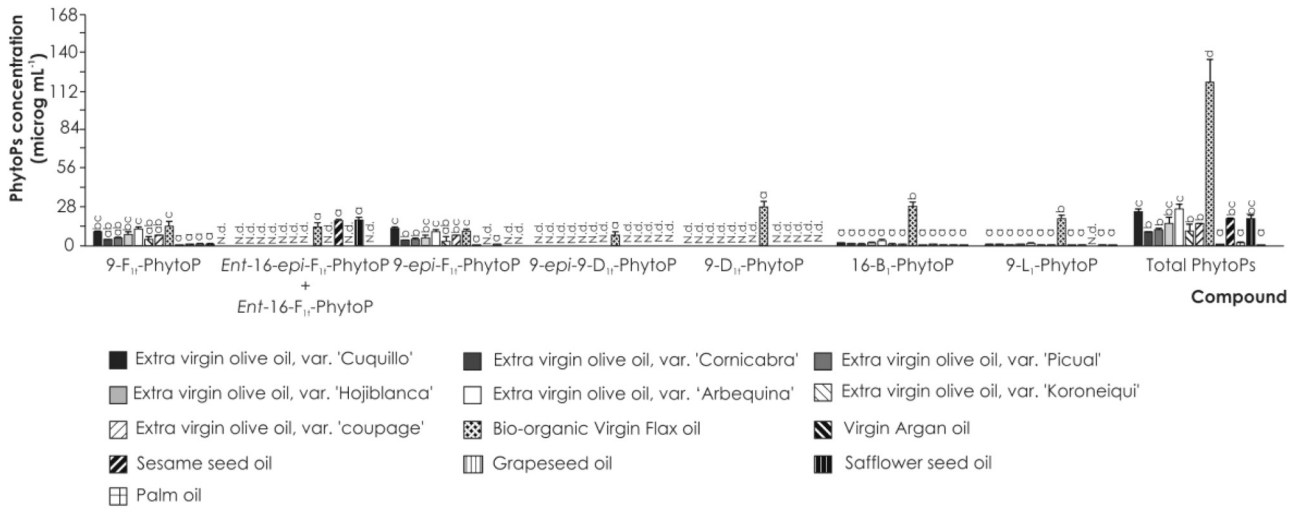


Fig. 2. Concentrations of the phytoprostanes (PhytoPs) 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP in 0.1° extra virgin olive oils made using the drupes varieties 'Arbequina', 'Coupage', 'Koroneiki', 'Cuquillo', 'Hojiblanca', 'Cornicabra', and 'Picual', and in bio-organic virgin flax, virgin sesame, virgin argan, safflower seed, grapeseed, and palm oils. Values in the bar plot are annotated as the mean ± SD (n = 3). Columns with different letters, for each PhytoP, are significantly different at $p < 0.05$ according to the ANOVA and Tukey's multiple range tests.

PhytoFs in tissues and organs, on their derivatives and esterified forms, and consequently on their potential biological interest. This should encourage the study of the bioavailability of PhytoPs and PhytoFs and their biological activity, essential to shed some light on the bioactive compound/s responsible for the contribution of plant foods to health and wellbeing in humans (Collado-González et al., 2017). However, to complete these studies, as a primary step, it is essential to select proper food matrices according not only to the plant oxylipins profile, but also the quantitative occurrence of such compounds.

The evaluation of the array of vegetable oils considered in the present work provided valuable information on the relative abundances of the separate PhytoPs and PhytoFs, and on their interest as dietary sources of such compounds. Hence, in the EVOOs the 9-series of F_1-PhytoPs were the most abundant (9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP), being found in concentrations ranging from 3.03 to 12.54 μg mL^{-1}. On the other hand, in flax oil, besides the high concentration of the 9-series of F_1-PhytoPs (14.02 and 10.56 μg mL^{-1}, respectively), D_1-, L_1-, and B_1-PhytoPs were found at concentrations of up to 28.09, 28.67, and 19.22 μg mL^{-1}, respectively (Fig. 2). The additional vegetable oils considered (argan, sesame, grapeseed, safflower, and palm) displayed amounts lower than the LOQ of the method - except for 9-F_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP, which were detected and quantified, although at much lower concentrations than in olive and flax oils (0.54, 0.25, and 0.10 μg mL^{-1}, on average, respectively). Sesame and safflower oils also exhibited relatively high concentrations of *ent*-16-F_{1t}-PhytoP and *ent*-16-*epi*-F_{1t}-PhytoP (17.85 and 18.34 μg mL^{-1}, respectively), which were at levels similar to those in flax oil (Fig. 2). These ranges of concentration are in agreement with those previously reported, with minor differences that could be attributable to the influence of the cultivar evaluated, the oil extraction technology, and/or storage conditions that make the oils prone to oxidation (Barbosa et al., 2015; Carrasco-del Amor et al., 2015; Carrasco-Del Amor et al., 2016; Carrasco-Del Amor et al., 2017; Collado-González, Moriana, et al., 2015; Karg et al., 2007).

Calculation of the total concentration of PhytoPs evidenced the preponderance of flax oil as a dietary source of these compounds with 119.15 μg mL^{-1}, on average - a value that surpassed 7.3-fold the level found in the diverse varieties of EVOO (16.31 μg mL^{-1}, on average) and 6.1-fold the levels in sesame and safflower oils (19.42 μg mL^{-1}, on average). By contrast, PhytoPs were almost absent in argan, grapeseed, and palm oils, with values lower than 1.6% of that recorded in flax oil (< 1.90 μg mL^{-1}) (Fig. 2).

These values are partially in agreement with those of Karg et al. (2007), who described the concentrations of PhytoPs in edible oils (linseed, olive, soybean, rapeseed, and walnut), discriminating the classes A_1-, B_1-, E_1-, and F_1-PhytoPs. In this work of Karg et al., the A_1-, B_1-, E_1-, and F_1-PhytoPs occurred in the concentration ranges 0.27–21.94 μmol L^{-1} (0.08–6.75 μg mL^{-1}), 0.07–7.77 μmol L^{-1} (0.02–2.39 μg mL^{-1}), 3.41–66.50 μmol L^{-1} (1.11–21.7 μg mL^{-1}), and 0.45–33.54 μmol L^{-1} (0.35–11.00 μg mL^{-1}), respectively, with concentrations of total PhytoPs ranging from 7.31 to 81.16 μmol L^{-1} (2.38–26.43 μg mL^{-1}) (Karg et al., 2007). In addition, Karg et al. reported the relative preponderance of the F-, D-, and B-PhytoP classes; this depended not only on the type of matrix considered, but also on the features of the production procedures, which was further demonstrated by additional work in the field (Barbosa et al., 2015; Carrasco-del Amor et al., 2015; Karg et al., 2007).

The concentrations described by Karg et al. (2007) are lower than those obtained in the present work. Indeed, the concentration of total PhytoPs in our specific batch of olive oil samples (16.31 μg mL^{-1}, on average) is 7-fold greater than that reported by Karg et al. These differences cannot be explained only by the analytical methodology applied; the genetic features of the cultivars evaluated, the agroclimatic conditions to which the olive tree crops are exposed, and the technological settings used to obtain the edible oils should be considered also (Carrasco-Del Amor et al., 2016; Carrasco-Del Amor et al., 2017; Collado-González et al., 2016; Collado-González, Moriana, et al., 2015; Collado-González, Pérez-López, et al., 2015; Imbusch & Mueller, 2000a; Imbusch & Mueller, 2000b; Karg et al., 2007; Sattler et al., 2006; Thoma, Krichke, Loeffler, & Mueller, 2004). The impact of the technological settings on the final concentrations of PhytoPs and PhytoFs in vegetable oils is crucial to the selection of these food matrices for nutritional trials. In this sense, independently of the initial concentrations in foodstuffs, manufactured food needs to be further assessed.

The relative abundance of distinct PhytoPs classes cannot be compared among the diverse types of oils since it would be conditioned by specific physiological and molecular aspects; this would require equal matrices. For instance, flax oil has a preponderance of the 9-series of D_1- and L_1-PhytoPs, whilst the olive oils, in agreement with Karg et al. (2007), show a dominance of the 9-series of F_1-PhytoPs. These differences, that could be due to the PUFAs profile of the separate matrices (since not all vegetable oils contain equal concentrations of ALA), should be taken into consideration when it is intended to study the specific biological activity of separate series of PhytoPs (Collado-

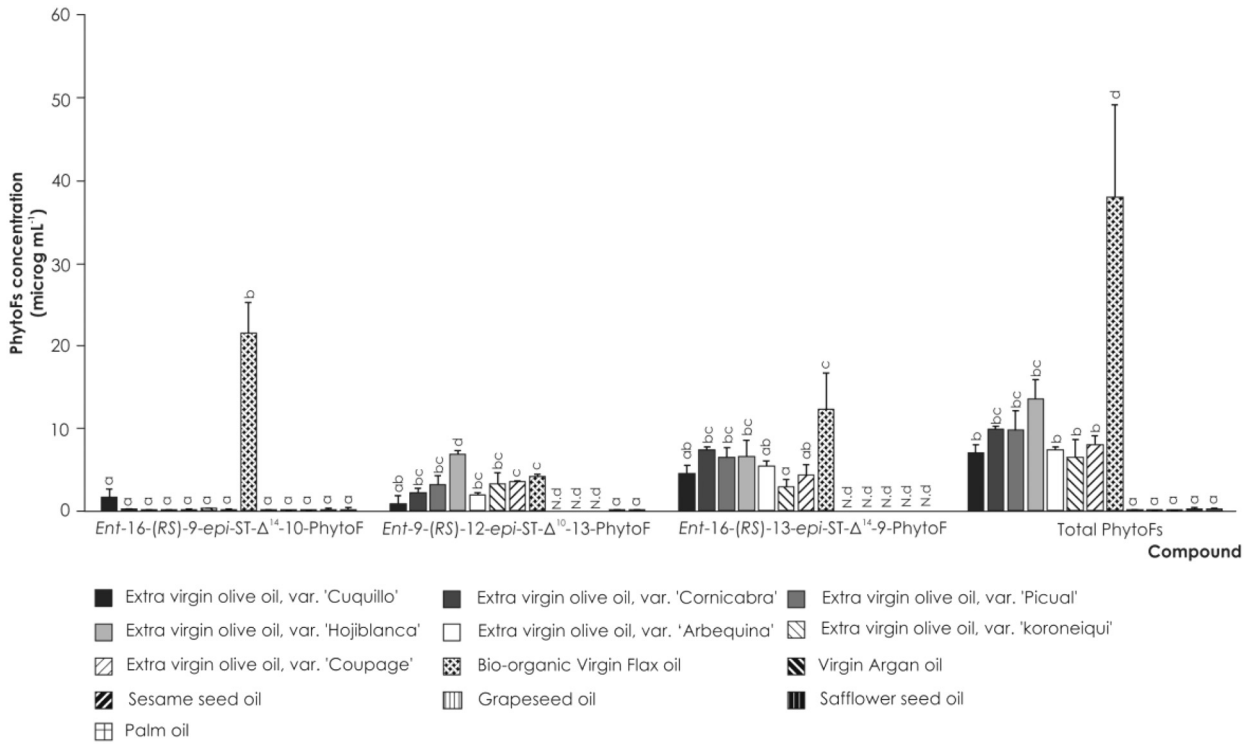


Fig. 3. Concentration of the phytofurans (PhytoFs) *ent*-16-(*RS*)-9-*epi*-*ST*-Δ^{14}-10-PhytoF, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in 0.1° extra virgin olive oils made using the drupes varieties 'Arbequina', 'Coupage', 'Koroneiki', 'Cuquillo', 'Hojiblanca', 'Cornicabra', and 'Picual', and in bio-organic virgin flax, virgin sesame, virgin argan, safflower seed, grapeseed, and palm oils. Values in the bar plot are annotated as the mean ± SD (n = 3). Columns with different letters, for each PhytoF, are significantly different at $p < 0.05$ according to the ANOVA and Tukey's multiple range tests.

González, Moriana, et al., 2015). In this sense, it has been suggested that, under the physical conditions present during the olive oil production process, 16-B_1-PhytoP and 9-L_1-PhytoP could be released from the plant material into the covering liquid (Thoma et al., 2003).

To date, no information on the content of PhytoFs in vegetable oils has been reported. These compounds were detected in all 13 oils assessed in the present work, although with noteworthy differences among them. Thus, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF was the most abundant one in flax oil (21.46 μg mL^{-1}, on average), surpassing the concentrations in the seven varieties of EVOO, as well as those in argan, sesame seed, grapeseed, safflower, and palm oils (which had similar, lower values), by 98.9%, on average (Fig. 3). The concentration of *Ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF was highest in EVOO var. 'Hojiblanca' (6.87 μg mL^{-1}), with no significant differences between the remaining olive oils and flax oil (2.72 μg mL^{-1}, on average). Finally, with respect to *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, flax oil again displayed the highest concentration (12.28 μg mL^{-1}), although the statistical differences from the olive oils were dependent on the variety considered. Thus, whilst the concentrations in 'Cornicabra', 'Picual, and 'Hojiblanca' (6.80 μg mL^{-1}, on average) did not differ significantly relative to flax oil (4.27 μg mL^{-1}, on average), the concentrations in 'Cuquillo', Arbequina', 'Koroneiqui', and 'Coupage' were 65.2% (significantly) lower than in flax oil. *Ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF was not found in argan, sesame seed, grapeseed, safflower, or palm oil. The lowest concentration (or absence) of PhytoFs corresponded to argan, sesame seed, grapeseed, safflower, and palm oils (Fig. 3).

Similarly to PhytoPs, the highest concentration of total PhytoFs corresponded to flax oil (37.92 μg mL^{-1}) and surpassed the concentration of the seven varieties of EVOO (8.85 μg mL^{-1}, on average) by 76.7%. The PhytoFs were almost absent in the remaining oils, that exhibited values ranging from 0.03 to 0.26 μg mL^{-1} (Fig. 3).

Although to date it has been initiated the study of the properties of vegetable oils as dietary sources of PhytoPs, as far as we know, only a few scientific articles have reported the occurrence of PhytoFs, plant metabolites derived from ALA oxidation induced by ROS and formed as a consequence of the addition of molecular oxygen after the initial cyclization of ALA in the biosynthesis of PhytoPs in nuts and seeds, as well as in melon leaves (Cuyamendous et al., 2015; Yonny et al., 2015). Nonetheless, PhytoFs have been identified in nuts, which are characterized by a high concentration of PUFAs. In this regard, Cuyamendous et al. described the presence of *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in concentrations that did not correlate significantly with that of ALA (Cuyamendous et al., 2015). Moreover, these compounds have been found also in vegetable oils of diverse origin, in different concentrations - reinforcing the relevance of the genetic background to the PhytoFs profile in higher plants (Carrasco-del Amor et al., 2015).

Independent of the individual compounds present in the separate foodstuffs, the evident higher concentrations of PhytoFs found in vegetable oils should be considered carefully because of the influence of the production processes on non-enzymatic oxidation of PUFAs and, consequently, on the final concentration of PhytoFs.

3.6. Principal component analysis

After profiling and quantifying the PhytoPs and PhytoFs in vegetable oils, the data were examined further using PCA, a statistical technique that simplifies a complex data set by reducing the number of variables (Anuar, Taha, & Mahmad, 2015). This (theoretical) classification contributed to the identification of groups of vegetable oils according to their PhytoPs and PhytoFs profiles, thus indicating candidates for inclusion in future nutritional trials.

Hence, 12 components representing the total variance (100.0%) were extracted, with the first nine explaining > 99.9% of the variance, whilst the first two principal components (PCs) explained 87.7% (Table 5). Concomitantly, the weight of each variable (chemical parameter) for the PCs was determined; the set of these loadings constitutes an Eigenvector (Wold, Esbensen, & Geladi, 1987). Of the total variance, 70.2% was explained by PC1 (Table 5), being mostly attributable to 9-

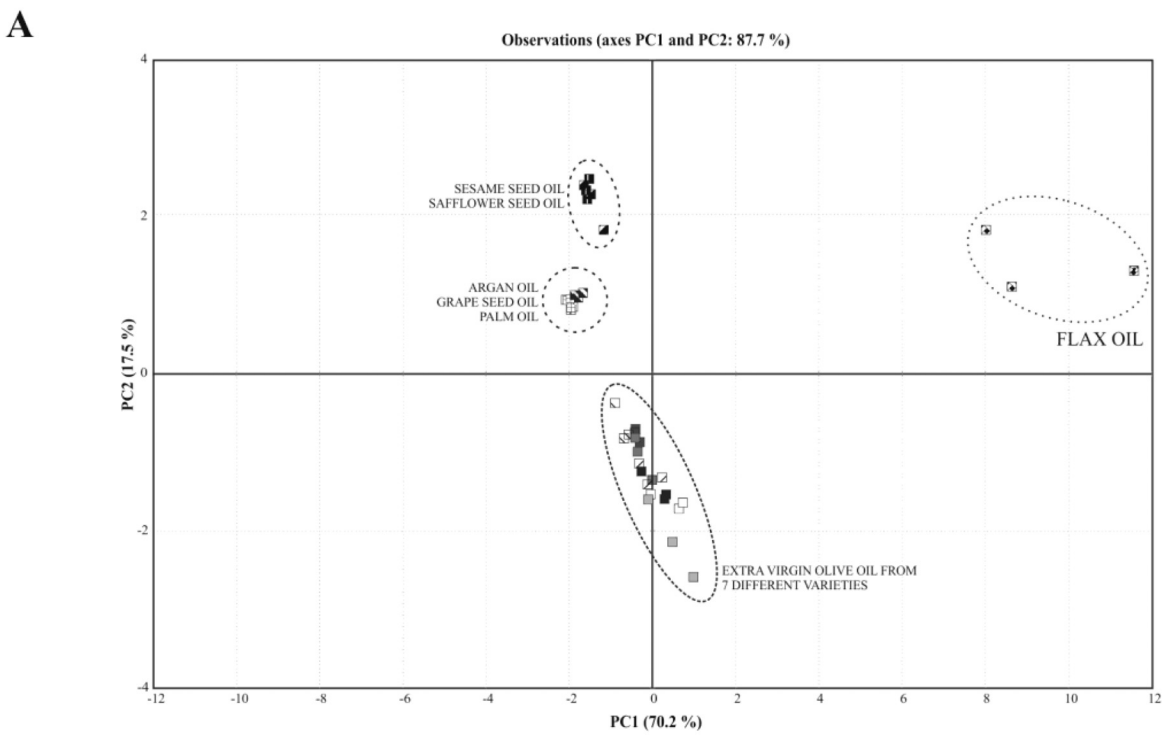
Table 5
Variance and cumulative variance provided by each principal component retrieved.

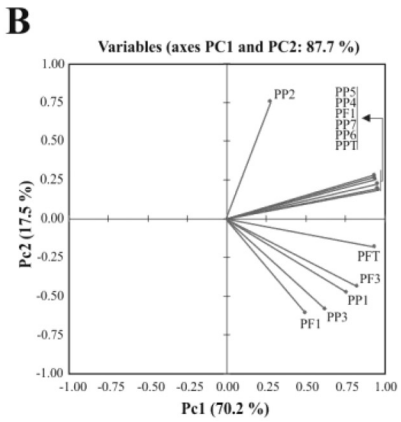
PC	Variance (%)	Cumulative variance (%)
1	70.2	70.2
2	17.5	87.7
3	5.7	93.4
4	4.4	97.8
5	1.2	98.9
6	0.6	99.5
7	0.4	99.9
8	0.1	> 99.9
9	< 0.1	> 99.9

epi-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP, 9-L_1-PhytoP, total PhytoPs, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, and total PhytoFs. With respect to PC2, the concentrations of 9-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF were the most relevant contributors. The percentage of the variance explained by PC2 was 17.5% (Table 5).

The PCA developed using the concentrations of PhytoPs and PhytoFs provided clusters alongside the PC1 *vs* PC2 plot (Fig. 4A). In this plot, the capacity of the PCA to discriminate the diverse matrices categorized according to the concentrations of these plant oxylipins was evident. Indeed, four clusters were clearly separated, with positive scores for PC1 (flax oil, -cluster 1-), negative scores for PC1 (argan, grapeseed, and palm oils -cluster 2- and sesame and safflower oils -cluster 3-), and intermediate (positive and negative) scores for PC1 (EVOOs, -cluster 4-). Regarding the ordinates axis (PC2), clusters 1–3 displayed positive scores, whilst cluster 4 had negative values.

The analysis of the samples distribution, jointly with the eigenvectors informing on the weight of each variable and the correlation between them, indicated the relationship of the observed classification with the abundance of compounds in the samples under evaluation. In this sense, the positive contribution of PhytoPs and PhytoFs to PC1 suggests a close correlation between the samples distribution and





Correlation matrix of Pearson's coefficients between individual and total phytoprostanes and phytofurans in vegetable oils.

Variables	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PPT	PF1	PF2	PF3
PP2	-0.023										
PP3	0.904**	-0.212									
PP4	0.561	0.392	0.409								
PP5	0.547	0.404	0.404	0.989***							
PP6	0.628*	0.360	0.484	0.982***	0.982***						
PP7	0.609*	0.376	0.460	0.994***	0.992***	0.996					
PPT	0.704**	0.470	0.562	0.954***	0.959***	0.968	0.970***				
PF1	0.576	0.388	0.450	0.993***	0.996***	0.982	0.993***	0.965***			
PF2	0.558	-0.212	0.447	0.305	0.304	0.353	0.336	0.343	0.299		
PF3	0.799**	-0.065	0.721**	0.668*	0.653	0.707	0.698**	0.712**	0.671*	0.718**	
PFT	0.748**	0.135	0.627	0.847***	0.855**	0.854	0.864***	0.868***	0.861***	0.668*	0.912***

The Pearson's coefficients indicate a significant correlation at $p<0.05$(*), $p<0.01$(**), and $p<0.001$(***).

Fig. 4. Principal Component Analysis (PCA) of the phytoprostanes (PhytoPs) and phytofurans (PhytoFs) composition of vegetable oils. Correlation scatter plot and Pearson's correlation matrix (A) and PCA score scatter plot (B) considering data on the concentrations of PhytoPs and PhytoFs in 0.1° extra virgin olive oils made using the drupes varieties 'Arbequina', 'Coupage', 'Koroneiki', 'Cuquillo', 'Hojiblanca', 'Cornicabra', and 'Picual', and in bio-organic virgin flax, virgin sesame, virgin argan, safflower seed, grapeseed, and palm oils. PP1, 9-F_{1t}-PhytoP; PP2, *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP; PP3, 9-*epi*-9-F_{1t}-PhytoP; PP4, 9-*epi*-9-D_{1t}-PhytoP; PP5, 9-D_{1t}-PhytoP; PP6, 16-B_1-PhytoP; PP7, 9-L_1-PhytoP; PPT, total PhytoPs; PF1, *ent*-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF; PF2, ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF; PF3, *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF; PFT, total PhytoFs.

clusters through the abscises axis, whilst the growing content of almost all individual PhytoPs and PhytoFs contributed to positive values along this axis (Fig. 4A and B). On the other hand, those variables contributing to PC2 negatively necessitate caution in the interpretation of the samples classification, since the vegetable oils achieving positive scores along the ordinate axis presented the lowest concentrations of the PhytoPs 9-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP, and 9-*epi*-9-F_{1t}-PhytoP and the PhytoFs *ent*-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, and *vice versa*.

4. Conclusions

The recently emerging interest in the biological activity of vegetable oxylipins is related to their structural analogy with those of mammalian origin (isoprostanes and prostaglandins, among others), to which have been attributed a number of biological activities (still to be defined in detail) that make them relevant potential bioactive compounds at the physiological level. The selection of dietary sources of PhytoPs and PhytoFs is a major issue that needs to be addressed for the proper design of nutritional trials - that require the identification of a variety of foods, from among those widely included in the eastern dietary habits, as sources of these promising compounds. Thus, the joint analysis of the raw data and the results obtained from the PCA indicates that flax oil constitutes the best dietary source of PhytoPs and PhytoFs, whilst argan, grapeseed, and palm oils (all grouped in cluster 3) should be considered the most proper negative controls to be included in nutritional trials aimed at clarifying the actual bioavailability of such compounds given the virtual absence of PhytoPs and PhytoFs in their composition. In this respect, because of the negative connotations of the saturated fatty acids profiles of palm, argan, and grapeseed oils, these should be the options of choice. Moreover, this information may allow the selection of matrices to be incorporated into *in vitro* assays designed to yield mechanistic information on the biological activity of PhytoPs and PhytoFs in diverse pathophysiological situations. The demonstration of valuable biological activities for these compounds will boost, in the short term, the development of further studies on their capacity to act as functional analogs of mammalian oxylipins, contributing to the maintenance of the consumers' health and the development of a new family of functional products as a response to current health demands.

Acknowledgements

This work was partially funded by the “Fundación Séneca de la Región de Murcia” Grupo de Excelencia 19900/GERM/15. RDP was supported by a Postdoctoral Contract (Saavedra-Fajardo 20024/SF/16) from the Agency for Science and Technology of the Región de Murcia (Spain). This work is included in the framework of the collaboration between the Spanish Research Council (CEBAS-CSIC) and CNRS-University of Montpellier as “Projets Internationaux de Cooperation Scientifique (PICS-2015-261141). We are grateful to Dr. David Walker (native English speaker) for his review of the English grammar and style of the current report.

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