Determination of very long-chain polyunsaturated fatty acids from 24 to 44 carbons in eye, brain and gonads of wild and cultured gilthead sea bream (*Sparus aurata*)

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Very long-chain (> C24) polyunsaturated fatty acids (VLC-PUFA) play an important role in the development of nervous system, retinal function and reproductive processes in vertebrates. Their presence in very small amounts in specific lipid classes, the lack of reference standards and their late elution in chromatographic analyses render their identification and, most important, their quantification, still a challenge. Consequently, a sensitive and feasible analytical methodology is needed. In this work, we have studied the effect of chain length, as well as the number and position of unsaturations (or double bonds) on the response of GC-APCI-(Q)TOF MS, to establish an analytical method for VLC-PUFA quantification. The developed methodology allows the quantification of these compounds down to $2.5 \times 10^{-3}$ pmol/mg lipid. The reduction of VLC-PUFA levels in lipid fractions of the organs from the herein sampled farmed fish suggesting a yet undetected effect on these compounds of high vegetable oil aquafeed formulations, that currently dominate the market.

Long-chain (C20–C24) polyunsaturated fatty acids (LC-PUFA) are regarded as very important compounds with physiologically critical functions in vertebrates including humans. Among them, the n-3 (or “omega-3”) eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n-3), and the n-6 arachidonic acid (ARA, 20:4n-6), are important for normal growth and development, and, in mammals, they play pivotal roles in the inflammatory response. Importantly, dietary consumption of EPA and DHA has been found beneficial in cardiovascular and cerebrovascular conditions, and some types of cancer. Marine fish, particularly oily species, represent a major source of n-3 LC-PUFA in the humans diet, and as a consequence, these compounds have been of special concern for researchers. Farmed fish represent an ever-increasing portion of fish consumed by humans. The rapid expansion of aquaculture in the last decades has urged the need to use non-fish derived raw materials in the feed. Consequently, aquafeed formulations have remarkably decreased the levels of LC-PUFA, hence impacting the nutritional value of the final product because of the reduced levels of the health-promoting n-3 LC-PUFA such as EPA and DHA, and interestingly, compromising the health of the farmed fish itself whereby LC-PUFA have critically important functions as described above for mammals.

LC-PUFA are biosynthetic precursors of the so-called very long-chain (> C24) PUFA (VLC-PUFA) and, therefore, the abovementioned reduction of LC-PUFA in fish diets has been hypothesized to compromise the physiological demands of VLC-PUFA in farmed fish. It is now well established that the elongation of very long-chain fatty acid protein 4 (Elov4) is a pivotal enzyme determining the bioconversion of LC-PUFA into VLC-PUFA. VLC-PUFA have been found in low concentrations in retina, sperm and brain of mammals, as a part of phosphatidylcholine (PC), sphingomyelin (SM) and cerebrosides (CE), and have been therefore considered essential for development of nervous system, retinal function and reproductive processes. Since their detection in bovine retinas by Aveldaño et al. in 1987, the study of VLC-PUFA has been mainly focused on mammals. Surprisingly, the detection and characterization of VLC-PUFA has been barely studied in...
Variation and confidence interval at 95% (n = 8) were 4.6% and ± 4.2%, respectively (Figs. S1, S2). In view of the above-mentioned absence of reference standards makes difficult the identification of VLC-PUFA, their quantitation, aggravated by their extremely low abundance pointed out above, is almost an unexplored field. Furland et al. expressed semi-quantitative results of VLC-PUFA content in rat testis as percentages of the total fatty acids in each lipid class. Méndez et al. and Liu et al. based the quantitative analysis of VLC-PUFA on the internal standard (IS) method by the determination of mass response factor, extrapolating the data from existing standards C30:0-C36:0. In the above studies, the extrapolation of the response factor did not take into account the dependence of the instrument response on chain length, number and position of unsaturations.

While the above-mentioned absence of reference standards makes difficult the identification of VLC-PUFA, their quantitation, aggravated by their extremely low abundance pointed out above, is almost an unexplored field. Furland et al. expressed semi-quantitative results of VLC-PUFA content in rat testis as percentages of the total fatty acids in each lipid class. Méndez et al. and Liu et al. based the quantitative analysis of VLC-PUFA on the internal standard (IS) method by the determination of mass response factor, extrapolating the data from existing standards C30:0-C36:0. In the above studies, the extrapolation of the response factor did not take into account the dependence of the instrument response on chain length, number and position of unsaturations.

Recent studies have applied APCI source with quadrupole time-of-flight mass analyzers (QTOF) coupled to GC to the qualitative analysis of VLC-PUFA in fish. This soft ionization technique has allowed to obtain the parent molecule, enabling the accurate identification of VLC-PUFA by means of measuring protonated molecule at accurate mass. Moreover, the study of the mass spectrometric behavior enables the discrimination between n-3 and n-6 compounds. The application of this technique has also allowed, for the first time, the identification of VLC-PUFA with chains up to 44 carbons in the SM and PC fractions in the total lipids from fish based on their accurate mass and fragmentation pattern.

The methodology established in this work is able to identify, and also quantify, VLC-PUFAs with chains up to 44 carbons in fish organs, improving previous methodologies taking advantage of the GC-APCI-QTOF, not used before to the best of our knowledge. The effect of chain length, number of unsaturations and their position to the response of GC-APCI-QTOF was studied in order to establish a quantification method of VLC-PUFA, taking into account the possible variations of the VLC-PUFA response respect PUFA standards available. This methodology was applied in the identification and quantification of VLC-PUFA known to be present in the PC, SM and CE lipid fractions of eye, brain and gonads of wild and culture gilthead sea bream (Sparus aurata), providing valuable information, for future studies in the fields of lipid biochemistry and aquaculture, about the presence of these novel molecules in wild and farmed fish tissues.

**Results and discussion**

**Identification of VLC-PUFA.** Like in a previous work, the identification of VLC-PUFA was conducted looking for a chromatographic peak for each of the corresponding [M+H]+ ions using 0.02 Da narrow window extracted Ion Chromatograms (nw-XICs). Mass spectra of the different VLC-PUFA peaks were studied for further identification of the compounds. The mass accuracy of [M+H]+ was evaluated in the low-energy (LE) function, and mass errors were below 3.2 ppm in all cases. Table S1 shows the mass spectrometry parameters used for the identification of targeted compounds (for more details see Serrano et al.).

**Quantification of VLC-PUFA.** In order to develop a quantification method based on Q-TOF mass analyzer with APCI source, slopes of calibration curves (n = 6) from equimolar mixtures (0.002, 0.02, 0.2, 2 and 4 pmol/µL) of each saturated FAME (18:0, 20:0, 22:0, 24:0, 26:0, 28:0 and 30:0) and PUFA methyl esters (20:3n-3, 20:3n-6, 20:4n-3, 20:4n-6, 20:5n-3, 22:2n-6, 22:4n-6 and 22:6n-3) were studied to elucidate the effect of chain length, as well as position and number of unsaturations, on the instrumental response. Comparison of slope values from saturated FAMEs and PUFA calibration curves, respectively, using Student's t-test based in both the standard error of the regressions and the standard error of the slopes revealed no statistical differences and no trends regarding length of chains and position and number of unsaturations (Fig. 1).

Slopes from FAMEs presented a variation coefficient of 5.6% with a confidence interval at 95% of ± 4.9% (n = 7). Regarding PUFA methyl esters, statistical analysis showed also that slopes were statistically equal and position and number of unsaturations did not show relation with instrumental response. Values of coefficient of variation and confidence interval at 95% (n = 8) were 4.6% and ± 4.2%, respectively (Figs. S1, S2). In view of the results, the points of the calibration curves for VLC-PUFA quantification were calculated as the arithmetic mean of the responses of each of the eight available PUFA standards (n = 6). Hence, the response obtained by GC-APCI-QTOF MS did not show appreciable trends related to the characteristics of the molecules, minimizing possible biases in the determination of very long chain molecules.

Table 1 shows the uncertainties for each point of calibration curve calculated as the arithmetic mean from eight calibration curves corresponding to each PUFA methyl ester reference standard available (see Fig. S1),
Figure 1. Slopes of the calibration curves from the responses of saturated fatty acids and PUFA methylated reference standards available. Error bars: standard deviation (n = 6).

Table 1. Uncertainties of the experimental calibration curve calculated as the arithmetic mean from eight calibration curves corresponding to PUFA methyl ester standards. *Calculated at 95% confidence level.

| Concentration of calibration point (pmol/µL) | Uncertainty (%)* | Uncertainty (pmol/µL) |
|--------------------------------------------|------------------|-----------------------|
| 0.002                                      | 5.5              | 0.0002                |
| 0.02                                       | 4.9              | 0.001                 |
| 0.2                                        | 4.3              | 0.002                 |
| 2                                          | 2.8              | 0.006                 |
| 4                                          | 4.05             | 0.08                  |
carbons detected and, interestingly, these C44 VLC-PUFA were only present in PC from brain of wild in selected lipid classes from gilthead sea bream organs. As it can be observed, molecules with chains up to 44 VLC-PUFA), C30–C38 (medium-chain VLC-PUFA) and C40–C44 (long-chain VLC-PUFA). Short VLC-PUFA are biosynthesized in fish through Elovl enzyme-mediated reactions from LC-PUFA precursors30, fish showed lower content of VLC-PUFA (t-test, 99% confidence level) than wild specimens. Considering that VLC-PUFA are biosynthesized in fish through Elovl enzyme-mediated reactions from LC-PUFA precursors30, these results suggest that diet can impact VLC-PUFA homeostasis and, potentially, the biological functions that these compounds exert in vision and reproduction. On one hand, natural diets of a carnivorous fish like the gilthead sea bream are rich in LC-PUFA and therefore, biosynthesis of VLC-PUFA guarantees the physiological demands in key tissues. On the other, compared to wild fish, dietary supply of LC-PUFA in farmed fish, albeit likely covering their minimum nutritional requirements, is largely lower due to the high inclusion levels of non-marine ingredients in current aquafeed formulations31. Thus provision of adequate levels of LC-PUFA in the diet affects more strongly eyes and gonads, being these organs more dependent on an adequate dietary provision of VLC-PUFA precursors (i.e. LC-PUFA)1,32–36 generally found in lower amounts in substituted diets15,33,37.

As can be observed in Fig. 2A, VLC-PUFA are especially abundant in the phosphatidylcholine fraction. Sphingomyelin and cerebrosides fractions present, in all organs analyzed, higher content of VLC-PUFA in the phosphatidylcholine fraction, in selected organs of wild and farmed specimens of gilthead sea bream (more details on VLC-PUFA concentrations in Tables S2 and S3). Quantitation shows that PC is the lipid class with the highest content of VLC-PUFA. Sphyngomiyelin and cerebrosides fractions present, in all organs analyzed, higher content of VLC-PUFA in the phosphatidylcholine fraction, in selected organs of wild and farmed specimens of gilthead sea bream (more details on VLC-PUFA concentrations in Tables S2 and S3). Quantitation shows that PC is the lipid class with the highest content of VLC-PUFA.

Table 2. Validation data of the proposed method at two levels of concentration for standards and internal standard (n = 5). rsd relative standard deviation.

| Standard | Retention time (min) | Concentration (pm/µL) | Recovery ± rsd (%) |
|----------|---------------------|-----------------------|--------------------|
| C28:0    | 8.98                | 0.02                  | 124 ± 7            |
| C30:0    | 9.58                | 0.02                  | 112 ± 8            |
| C27:0 (IS)| 8.67                | 0.25                  | 71 ± 9             |

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Reagents and solvents. In this research, commercially available fatty acids and PUFA analytical standards were selected. Free form and methylated (ME) saturated fatty acids from 18:0 to 24:0 were purchased from Fluka.

### Table 3. Concentrations of VLC-PUFA (pmol/mg Lipid) found in the eyes, brain and gonads samples from wild (W) and farmed (F) specimens of gilthead sea bream. (RSD (%) range: 6–40) (more details in Supplementary information).

| Compounds  | Eyes | Brain | Gonad |
|------------|------|-------|-------|
|            | SM   | PC    | CE    | SM   | PC    | CE    | SM   | PC    | CE    |
| 24:4n-6    | W 0.028 | 0.030 | 0.011 | 0.028 | 0.030 | 0.011 | 0.024 | 0.25  | 0.17  |
|            | F 0.005 | 0.05  | 0.005 | 0.005 | 0.05  | 0.005 | < LOQ | nd    | na    |
| 24:5n-6    | W < LOQ | 0.044 | 0.004 | 0.036 | 0.229 | 0.014 | 0.044 | 0.214 | 0.036 |
|            | F nd   | < LOQ | na    | 0.005 | 0.363 | 0.005 | < LOQ | 0.186 | na    |
| 24:6n-3    | W 0.010 | 0.143 | 0.006 | 0.083 | 0.372 | 0.022 | 0.174 | 0.475 | 0.030 |
|            | F nd   | 0.033 | na    | 0.005 | 0.937 | 0.005 | < LOQ | 0.544 | na    |
| 26:4n-6    | W 0.005 | 0.014 | < LOQ | 0.004 | 0.021 | 0.006 | 0.009 | 0.052 | 0.00  |
|            | F nd   | na    | < LOQ | 0.005 | nd    | nd    | nd    | nd    | na    |
| 26:5n-6    | W 0.006 | 0.032 | < LOQ | 0.014 | 0.066 | 0.009 | 0.013 | 0.057 | 0.010 |
|            | F nd   | na    | < LOQ | 0.005 | 0.121 | < LOQ | < LOQ | 0.059 | na    |
| 26:6n-3    | W < LOQ | 0.034 | < LOQ | 0.066 | 0.046 | 0.008 | 0.011 | 0.030 | nd    |
|            | F nd   | na    | < LOQ | 0.005 | 0.005 | < LOQ | < LOQ | < LOQ | na    |
| 28:4n-6    | W 0.008 | 0.014 | < LOQ | 0.006 | 0.010 | 0.002 | 0.007 | 0.022 | nd    |
|            | F nd   | na    | < LOQ | 0.005 | nd    | < LOQ | nd    | na    | na    |
| 28:5n-3    | W < LOQ | < LOQ | < LOQ | 0.006 | 0.010 | 0.009 | 0.007 | 0.012 | 0.011 |
|            | F nd   | nd    | na    | 0.005 | 0.005 | < LOQ | nd    | na    | na    |
| 28:6n-3    | W < LOQ | 0.042 | < LOQ | 0.007 | 0.029 | 0.004 | 0.008 | 0.018 | nd    |
|            | F nd   | na    | < LOQ | 0.005 | 0.005 | nd    | < LOQ | na    | na    |
| 30:4n-6    | W 0.020 | 0.040 | < LOQ | 0.006 | 0.009 | nd    | 0.007 | 0.021 | nd    |
|            | F nd   | na    | < LOQ | 0.005 | 0.004 | < LOQ | nd    | na    | na    |
| 30:5n-3    | W 0.040 | 0.114 | 0.005 | 0.007 | 0.016 | nd    | 0.010 | 0.042 | nd    |
|            | F nd   | na    | < LOQ | 0.005 | 0.005 | nd    | < LOQ | nd    | na    |
| 30:6n-3    | W 0.007 | 0.261 | 0.05  | 0.008 | 0.022 | nd    | 0.013 | 0.023 | nd    |
|            | F nd   | 0.052 | na    | 0.004 | 0.005 | < LOQ | < LOQ | nd    | na    |
| 32:4n-6    | W 0.006 | 0.046 | < LOQ | < LOQ | 0.006 | nd    | 0.007 | 0.019 | nd    |
|            | F nd   | na    | na    | nd    | nd    | nd    | nd    | nd    | na    |
| 32:5n-3    | W 0.016 | 0.306 | 0.006 | 0.006 | 0.012 | nd    | 0.012 | 0.028 | nd    |
|            | F nd   | 0.021 | na    | 0.004 | 0.005 | nd    | < LOQ | nd    | na    |
| 32:6n-3    | W 0.100 | 0.500 | 0.071 | 0.026 | 0.033 | 0.006 | 0.033 | 0.059 | 0.01  |
|            | F 0.015 | 0.516 | na    | 0.005 | 0.005 | < LOQ | < LOQ | nd    | 2Na   |
| 34:4n-6    | W 0.004 | 0.006 | nd    | < LOQ | 0.009 | nd    | 0.007 | 0.018 | nd    |
|            | F nd   | na    | < LOQ | < LOQ | nd    | nd    | nd    | nd    | na    |
| 34:5n-3    | W < LOQ | 0.012 | < LOQ | 0.006 | 0.013 | nd    | 0.008 | 0.039 | nd    |
|            | F nd   | nd    | na    | 0.005 | 0.005 | nd    | < LOQ | < LOQ | na    |
| 34:6n-3    | W 0.010 | 0.118 | < LOQ | 0.008 | 0.027 | 0.004 | 0.027 | 0.037 | nd    |
|            | F nd   | 0.019 | na    | 0.005 | 0.005 | nd    | < LOQ | < LOQ | na    |
| 36:6n-3    | W 0.006 | 0.017 | < LOQ | 0.0133 | 0.055 | < LOQ | 0.010 | 0.026 | nd    |
|            | F nd   | nd    | na    | 0.005 | 0.005 | nd    | < LOQ | < LOQ | na    |
| 38:6n-3    | W < LOQ | 0.008 | < LOQ | 0.022 | 0.135 | 0.002 | 0.007 | 0.019 | nd    |
|            | F nd   | na    | na    | 0.005 | 0.007 | nd    | < LOQ | < LOQ | na    |
| 40:6n-3    | W 0.006 | 0.021 | < LOQ | 0.004 | 0.423 | 0.007 | nd    | 0.016 | nd    |
|            | F nd   | na    | na    | 0.005 | 0.153 | < LOQ | na    | < LOQ | na    |
| 42:6n-3    | W nd   | < LOQ | nd    | 0.007 | 0.020 | nd    | nd    | nd    | nd    |
|            | F nd   | na    | < LOQ | nd    | nd    | nd    | nd    | nd    | na    |
| 44:6n-3    | W nd   | nd    | nd    | 0.004 | nd    | nd    | nd    | nd    | na    |
|            | F nd   | nd    | nd    | nd    | nd    | nd    | nd    | na    | na    |
PUFA including 20:4n-3, 20:4n-6, 20:5n-3, 22:4n-3, 22:4n-6, 22:5n-3, 22:6n-3 and 24:5n-3 standards were purchased from Supelco (Bellefonte, PA, USA). Stock solutions (around 500 μg/mL, except 1 μg/mL for 24:0-ME and 26:0-ME) were prepared by dissolving solid reference standards in hexane or by diluting reference standard solutions in hexane and subsequently stored in a freezer at –20°C under a N₂ atmosphere. Working solutions were prepared by diluting stock solutions in hexane.

Hexane (ultra-trace quality) was purchased from Scharlab (Barcelona, Spain). Chloroform (CHCl₃), diethyl ether and toluene were purchased from Merck Millipore (Darmstadt, Germany). Methanol (MeOH) was purchased from VWR (Radnor, PA, USA). Sulfuric acid, glacial acetic acid and potassium chloride (KCl) were purchased from Panreac (Castellar del Vallès, Barcelona, Spain). Iodine and butylated hydroxytoluene (BHT, content > 99%), used as antioxidant, were purchased from Sigma Aldrich (St. Louis, MO, USA). All reagents were analytical grade. HPLC-grade water was obtained by purifying double distilled water in a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA) (for more details see Garlito et al. 22).

**Instrumental.** An Agilent 7890 N gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a quadrupole orthogonal acceleration time-of-flight mass spectrometer, Xevo G2 QTOF (Waters Corporation, Manchester, UK), equipped with APGC v2.0 as ionization source, working in positive APCI mode. A fused silica VF-5HT capillary column with a length of 15 m × 0.32 mm i.d. and a film thickness of 0.10 μm (J&W Scientific, Folson, CA, USA) was used for GC separation. The oven temperature was programmed as follows: 130 °C (1 min); 20 °C/min to 340 °C; (2.5 min) with a total runtime of 14 min. Pulsed splitless (25 psi) injections of 1 μL were carried out at 280 °C with a splitless time of 1 min. Helium 99.999% (Praxair, Spain) was used as carrier gas at a flow of 4 mL/min.

The interface and ionization source temperatures were set to 340 °C and 150 °C, respectively. N₂ was used as auxiliary gas at 200 L/h, as cone gas at 5 L/h and as make-up gas at 300 mL/min. The APCI corona discharge pin was operated at 1.7 μA and the cone voltage was set to 20 V.

The QTOF was operated at 2.5 spectra/s acquiring the mass range m/z 50–1000. The TOF MS resolution was approximately 15,000 (FWHM) at m/z 264. Acquisition was done in MSE mode in which two alternating acquisition functions with different collision energies were generated: the low-energy (LE) function, selecting a collision energy of 4 eV to avoid or minimize fragmentation, and the high-energy (HE) function, with a collision energy ramp ranging from 25 to 40 eV to obtain a greater range of fragment ions.

Heptacose (Sigma Aldrich, Madrid, Spain) was used for the daily mass calibration. Internal calibration was performed using octafluoronaphthalene (Sigma Aldrich, Madrid, Spain) as lock mass (monitoring the molecular ion, m/z 271.9872).

**Figure 2.** Concentrations (pmol/mg Lipid) of VLC-PUFA determined in lipid classes contained in eye, gonads and brain of wild and farmed specimens of gilthead sea bream. SM sphingomyelin, PC phosphatidylcholine, CE cerebrosides.
In order to work under proton transfer conditions, an uncapped vial containing water was placed in a designed holder into the APCI source door to enhance protonation. MS data were acquired in centroid mode and processed by the ChromaLynx XS application manager (within MassLynx v4.1; Waters). Mass-Fragment software (Waters) was used for mass spectra interpretation.

**Sampling and sample treatment.** Eyes, brains and gonads (n > 5) were collected from wild (western Mediterranean) and farmed specimens of gilthead sea bream from local (Castellón, Spain) markets. Adult specimens of gilthead sea bream were dissected and eyes, brains and gonads were collected and stored at −20 °C until further analysis. Selection of organs and lipid classes was based on previous evidences of the presence of VLC-PUFA. Crystalline lenses were removed from eyes. Total lipids were extracted using the method of Folch et al. Subsequently, an aliquot of total lipids (~ 200 mg) was further developed by thin layer chromatography (TLC 20 × 20 silica gel G60, Merck, Darmstadt, Germany) using a polar solvent system (methyl acetate:propan-2-ol:chloroform:methanol:0.25% (w/v) aqueous KCI (25:25:25:10:9 by vol). The three fractions where VLC-PUFA were detected in previous studies, namely SM, PC and CE, were scraped off the plate, eluted in chloroform:methanol (2:1, v/v) containing BHT (0.01%, w/v), and used to prepare FAMEs. FAME samples were stored in hexane containing BHT (0.01%) under nitrogen at -20°C (for more details see Garlito et al. Prior to the analysis by GC-APCI-QTOF MS and GC-APCI-IMS-QTOF MS samples were dried with a gentle flow of N2 and reconstituted in 50 µL of hexane.

**Quantification of VLC-PUFA.** A direct calibration with internal standard (IS 27:0) calculated as the arithmetic mean of the responses of each of the eight available PUFA standards was used to quantify VLC-PUFA. Calibration curves were made by triplicate with five concentrations (0.002, 0.02, 0.2, 2, 4 pmol/µL). Internal standard was added to standards and samples before injection. Goodness of the regressions were checked by means of residuals study. Trends were not observed and residuals values were below 5% in all cases. Values of r² were higher than 0.99 in all cases. Homoscedasticity of the slopes were confirmed by means of F-test. The limit of detection (LOD) was estimated from the chromatograms of sample extracts fortified at the lowest level tested (i.e., 0.002 pmol/µL) for a signal-to-noise ratio of 3. LOQ was calculated as the concentration for a signal-to-noise ratio of 10.

**Validation.** In order to verify quantitatively the whole procedure, samples of eyes were fortified with the saturated FA standards with greater number of carbons (28:0 and 30:0), and with the IS used (C27), with the aim of simulating the VLC-PUFA molecules as best as possible. Recovery experiments (n = 5) at two different levels for 28:0 and 30:0 (0.02 and 2 pmol/µL), and at 0.25 pmol/µL for 27:0 (concentration added of IS), were performed. The fortified samples were left to stand for 1 h prior to extraction. Precision, expressed as relative standard deviation (%) was calculated from five replicates processed in the same conditions.

**Data processing.** Calculations and statistical tests were carried out using MS EXCEL 2013 (data analysis module). Slopes were statistically compared among them using Student’s t-test based in both the standard error of the regressions and the standard error of the slopes.

**Data availability** All data is available in the main text or the supplementary materials (contact with Roque Serrano, e-mail: serrano@uji.es. Corresponding author).

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