

Proposal for a fast method to determine routinely 15 plasticisers in olive oil by liquid extraction and Ultra High-Performance Liquid Chromatography Heated Electro Spray Ionisation High Resolution Mass Spectrometry (Orbitrap) analysis

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The aim of this research was to set up a fast and reliable analytical procedure to quantify 15 plasticisers among the most reported in olive oils, for routine controls addressed to laboratories of olive oil producer companies. The proposed procedure is a liquid-liquid extraction (it takes about 15 min including all handlings, and 1 ml of solvent used per sample) followed by UHPLC-HESI-HRMS (Orbitrap) analysis. A reverse phase, gradient separation on C18 column performs separation in about 12 min followed by column reconditioning. LOD, LOQ, trueness, linearity fit, repeatability (at 95% level of confidence) were also evaluated with satisfactory results. LODs range from 0.01 to 0.79 mg/kg; LOQs range from 0.05 to 2.63 mg/kg, whereas trueness ranges from 79 to 120%. Repeatability is 0.2 mg/kg for most of them at the concentration of interest (1.5 mg/kg), whereas it is 0.5-0.6 mg/kg for those that are unresolved mix of isomers (diisodecyl phthalate, DIDP; diisononyl phthalate, DINP). Accordingly, repeatability ranges from 0.2 to 0.6 mg/kg. Relevant coefficients of determination (R^2) were also determined and ranged between 0.96 and 0.99. As a conclusion, we can state that this method is a convenient procedure to analyse plasticisers in olive oil in routine controls.

Keywords: Plasticisers, Olive oil, Liquid-liquid extraction, Ultra high-pressure liquid chromatography, High-resolution mass spectrometry, Orbitrap

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1. INTRODUCTION

It is well known that plasticisers give plastics specific mechanical properties and are not bound to plastic polymer: this allows them to easily migrate from plastics into other materials they are in contact with. Phthalates (phthalic acid esters) are the most used compounds for that purpose, but also adipates, sebacates etc. are used in plastic manufacturing. Some of them are toxic to human health and specific migration limits (SML) from plastics have been established [1]. Research for suitable less toxic plasticisers are underway, but as plastics are extremely widespread all over the world, related human exposure has become a concern for all health safety boards. The European Food Safety Agency (EFSA) issued a series of opinions on different plasticisers and set TDI (Total Daily Intake) for many of them: e.g., for DEHP (bis-(2-ethylhexyl) phthalate), TDI is 0.05 mg/kg body weight [2]. In 2019, EFSA issued an update on five phthalates used in plastics intended for food contact and set new limits of safety in terms of TDI [3]. The European Chemical Agency (ECHA) recently issued [4] an Annex in force as of July 7th, 2020 to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) on 'Conditions of restriction' on the manufacture containing four phthalates, namely DEHP, DBP, BBP and DIBP.

As to the risk of cancer for humans, the International Agency for Research on

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Cancer (IARC) [5] classified DEHP in the Group 2B (possibly carcinogenic to humans).

US Environmental Protection Agency (EPA) [6] issued a list of priority pollutants related to the Clean Water Act that includes some common phthalates. Plasticisers as food contaminants fostered an endless number of scientific papers that cannot be reviewed here, but it is clear that it is a concern. Hereafter we mention just a few of them dedicated to vegetable oils, as an indication for reader. For vegetable oils, a direct immersion solid phase micro extraction (DI-SP-ME) from an extraction solvent (acetonitrile) and triple quadrupole MS for separation, identification and quantification was successfully used [7].

Different approaches to the determination of 16 phthalate esters in sesame oil were also used [8]. The authors chose and optimised a solvent extraction, solid-phase extraction (SPE) clean up and LC-MS/MS separation, identification, and quantification with deuterated internal standard. Different countries performed wide screenings on plasticiser occurrence in foods, household items and in the environment over the last 20 years. A recent investigation on phthalates in olive oil from the European market [9] showed DEHP and DINP as the most frequent phthalates occurring in that matrix. References to phthalate occurrence in vegetable oils from different countries were also provided. An investigation on phthalate contamination of vegetable oils marketed in Italy was performed using a GC-MS analytical method by direct injection into a programmed temperature vaporizing (PTV) of diluted oils [10]. Olive-derived oils resulted as more contaminated than other vegetable oils, mainly by DINP and DEHP. A survey performed on the occurrence of 10 plasticisers in food samples from Japan retailers [11] reported a lower contamination respect to just few years before. Authors adopted an HRGC/MS-SIM technique. Milk and products thereof were the most contaminated among vegetable oils, milk, yoghurt, and lard sold on the Italian market, as resulting from an investigation on phthalate contamination in those foods [12]. Even in this case, authors used HRGC/MS-SIM or Full Scan. Phthalates occurrence in the olive oil production chain, from the field to the oil mill storage was carried on in Friuli (NE Italy) [13]. The analytical technique consisted in the direct injection into PTV port of a GC-MS (SIM) of n-hexane oil solution, with injector back flush. The author finds and quantifies levels of phthalate at each stage of the production chain and examines all possible sources of contamination.

An HPLC-ESI-MS/MS technique to separate and identify seven phthalates in vegetable oils was validated with satisfactory results [14]. Chinese researchers performed investigations on plasticisers in different foods and oils sold on the local markets. Regarding commercial milk products, they chose an HPLC-ESI-MS-MS separation and quantification of 9 phtha-

lates [15]. They performed a solvent extraction with following steps of mixing, sonication, centrifugation, evaporation etc., before having the solution to inject. Results were satisfying in terms of precision and accuracy. A rapid, sensitive, and robust detection of phthalates in foods by GC/MS after solid phase extraction, or LC/MS after solvent extraction, sonication etc. with a noticeable use of solvents was presented by a Taiwanese researcher [16].

Different oilseeds (16) cultivated in China [17] showed an average contamination of 0.99 mg/kg. Authors used deuterated plasticisers as standards and HRGC-MS technique. Five phthalates in vegetable oils analysed by HRGC-MS were the subject of a recent research [18]. Authors studied samples coming from Zhengzhou (Henan, China): two of them were olive oils, and they found a maximum contamination around 1 mg/kg.

Chinese population exposure to phthalates was studied in the frame of the 5th Chinese Total Diet Study [19]. They considered 192 composite foods where they found contamination in about 76% of them, but never at serious levels.

Contamination of 21 vegetable oils with 15 plasticisers at the US retailer level was investigated [20]. They used HRGC-MS and compared EPA reference values with their results that were well below them. They concluded that no adverse effects on consumer health were recognised. Headspace SPME (PDMS fibre) extraction technique followed by HRGC-MS separation was also used to analyse vegetable oils [21]. A reliable method for seven plasticisers in alcoholic beverages from the US market based on UHPLC-MS/MS was set up along with determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) with satisfactory performance [22].

Phthalates in sediments and biota of an urbanised marine inlet near Vancouver (Canada) were determined [23]. They also proved LC-ESI-MS/MS technique as a suitable one to identify and quantify the mix of isomers from C6 to C10.

It is clear now that methods for the plasticiser determination in different matrixes have been used and described in many scientific papers, mainly devoted to the phthalates quantification in order to evaluate their impact on public health. The Joint Research Centre performed a survey on methods in use [24]: the most used was a liquid/liquid matrix extraction, followed by a clean-up performed by liquid-liquid partitioning or gel permeation chromatography (GPC). Separation and quantification were mainly carried out by HRGC equipped with mass detectors (SIM mode with single or triple quadrupole) or, hardly ever by HRGC-FID or -ECD. In few cases, liquid chromatography was also chosen (LC-ESI-MS/MS) with reliable results. A recent paper presents an up-to-date survey on the different analytical methods published so far for phthalate in

food samples [25]. The most common techniques were solvent- or sorbent-based extraction followed by GC or LC analysis. They also pointed out at least a couple of papers where the authors used capillary electrophoresis (CE).

This study aimed at developing a fast and reliable method for quantifying 15 plasticisers in olive oils: they are phthalates, adipates, citrate and sebacates. Routine controls require fast procedures with sample handlings as little as possible to lower contamination from glassware and laboratory environment [26]: solvent extraction from olive oils in thermally conditioned glassware, centrifugation, and supernatant injection into an UHPLC-HESI-MS/MS (Orbitrap) was performed. LOD (Limit of Detection), LOQ (Limit of Quantification), trueness (as a relative spike recovery around the concentration of interest 1.5 mg/kg each), repeatability limit at 1.5 mg/kg each (at 95% confidence level), were evaluated according to the Eurachem Guide [27]. It is important to remind that referring to the Eurachem Guide means evaluating the procedure 'fitness for purpose' without the need to make comparisons with other methods.

2. MATERIALS AND METHODS

2.1 SAMPLES

Olive oils were 30, ten from each of the following categories: extra virgin, lampante and refined olive oil available on the European market.

2.2 CHEMICALS

All solvents were of HPLC grade or LC-MS grade for mobile phase. Ammonium formate and formic acid for mobile phase buffering were of LC/MS grade (VWR, Pennsylvania, USA).

Single plasticisers were of purity $\geq 97\%$; pooled plasticiser standard solutions (at 1.5, 3.0 and 6.0 mg/l in acetonitrile) and some single compound solution (1.0 mg/l in acetonitrile) were certified by UltraScientific Italia (Anzola dell'Emilia, Bologna, Italy).

The solution used for oil extraction was acetonitrile saturated with n-hexane. n-Hexane was previously treated with aluminium oxide active basic, activity I, particle size 0.063-0.200 mm (70-230 mesh ASTM) for column chromatography (EMD Millipore, Massachusetts, USA) to adsorb any existing plasticiser contamination. Internal standard (IS) was triphenyl phosphate (TPP, 500 $\mu\text{g}/\text{mL}$ in MtBE, Dr Ehrenstorfer GmbH, Augsburg, Germany) diluted 1:100 with n-hexane saturated acetonitrile to have 5 $\mu\text{g}/\text{mL}$ working solution.

2.3 GLASSWARE

All glassware (10 ml test tubes, Pasteur pipettes, round flasks, 300 μL vials etc.) that need to be placed in contact with oils, solvents etc. must be conditioned at

400°C overnight before use and stored in closed conditioned glass jars. Aluminium foil to isolate cap septum from solution inside vials must be conditioned and stored in the same way.

2.4 UHPLC-HESI-HRMS (ORBITRAP)

An Accucore aQ, C18 column was used (2.6 μm , 2.1 \times 100 mm, ThermoScientific, Waltham, MA, USA, flow rate 0.300 mL/min) equipped with a Guard column (Universal Uniguard Holder with Defender guard, ThermoScientific). Separations were performed on a Dionex UltiMate 3000 UHPLC ThermoScientific, equipped with a four channels pump, autosampler and column compartment, both thermostatted at 15°C.

Mobile phase was made of A = water + 2% methanol + 0.1% formic acid + ammonium formate 5 mM, and B = methanol + 2% water + 0.1% formic acid + ammonium formate 2mM. A linear gradient separation was used as following: 0.0 - 4.5 min 25% A; 4.5 - 6.0 min, to 0% A; 6.0 min to 13.5 min 0% A; 13.5 min to 13.6 min to 25% A; 13.6 min to 15 min 25% A. Injection volume was 3 μL . HESI (Heated-Electro Spray Ionisation, Thermo Scientific) settings were the followings: sheath gas, nitrogen, 40 (arbitrary units); aux gas flow, nitrogen, 10 (arbitrary units); sweep gas flow rate: 0 (arbitrary units); Spray voltage, 3.50 kV; capillary temperature, 320°C; S-lens RF level: 55.0 (arbitrary units); aux gas heater temperature, 250°C. The HESI alignment must be set to maximise the response. In our case, the setting was: probe depth, C; front-to-back position, 1.75; side-to-side position, 0.

Mass detector was a Q Exactive Focus (ThermoScientific), operated in Full MS mode (mass range 190 to 455 m/z, Resolution = 70000; AGC target = 1e6; mass tolerance = 5 ppm) and PRM mode (Resolution = 17500; AGC target = 5e4, Isolation window = 3.0m/z, CE = 30); the Inclusion list contains 15 H⁺-plasticiser and Internal Standard adducts.

Computer programs for the acquisition and result processing were the following: Thermo TraceFinder EFS LC, Version 4.1; Tune file = Q Exactive Focus 2.8 SP1; Chromeleon Xpress, Version 6.80 SR13; Thermo Xcalibur, Version 4.0.27.19.

Table I lists the names and some data related to each plasticiser considered with the Internal Standard (TPP) also included.

It should be noted that, in our case, the most intense MS² TBC (tributyl-O-acetylcitrate) fragment is m/z = 129.01824. whereas it is commonly reported as less intense than m/z = 283.26422. Of course, we considered the intensity order as it came out from the instrument we used in this work.

2.5 CALIBRATION

Instrument calibration was performed using standard solutions at 1.5, 3.0 and 6.0 mg/l in acetonitrile with triple injection per level and excluding the zero point in

the regression. Of course, no matrix effect was considered at this time since plasticiser extraction from olive oil was under investigation.

2.6 OIL EXTRACTION AND SAMPLE PREPARATION FOR INJECTION

Weight an exact amount of about 0.1 g of oil into a glass tube using a Pasteur pipette; add 100 µl of IS solution (TPP at 5 µg/mL) and vortex it for about 1 min at the maximum speed. Add 900 µl of n-hexane saturated acetonitrile and vortex it for 1 min at the maximum speed, then cover the tube with aluminium foil and centrifuge it at 4000 rpm for 10 min. Transfer a supernatant aliquot into a 300 µL-conditioned vial. Crimp a cap after placing a conditioned aluminium foil between the vial top and the cap septum and then run the chromatographic separation.

3. RESULTS AND DISCUSSION

3.1 ANALYTICAL PERFORMANCE

LOD, LOQ, trueness and repeatability were evaluated. Table II shows the method performance parameters calculated on a basis of 10 *ab initio* independent replicates.

LOD determination was performed by spiking 10 replicates of oil blank at 3 levels of concentration each: 0.023 0.045 and 0.090 mg/kg. The oil blank was also checked for possible contaminations to be subtracted from the spiked oils. The 3-above level of concentrations were chosen after injection of consecutive dilutions of the pooled plasticiser standard solution in solvent (acetonitrile): the highest calculated concentrations that deviated for more than 20% from the

Table I - Plasticisers, CAS number, Retention time, Adduct, Formula, Adduct mass (m/z), Collision energy (eV), experimental MS² Fragments of all analyzed plasticisers

Plast icizer	CAS number	Rt (min)	Adduct	Formula	Adduct mass (m/z)	Collision energy (eV)	Fragment 1 (m/z)	Fragment 2 (m/z)
DMP	131-11-3	1.70	+H	C10H10O4	195.06519	70	163.03848	
DEP	84-66-2	2.06	+H	C12H14O4	223.09649	80	149.02337	
DPP	131-16-8	2.93	+H	C14H18O4	251.12779	35	149.02337	
TPP (IS)	115-86-6	3.72	+H	C18H15O4P	327.07807	35	233.03580	153.06955
DIBP	84-69-5	4.59	+H	C16H22O4	279.15909	80	65.03937	
BBP	85-68-7	4.85	+H	C19H20O4	313.14344	10	91.05420	149.02340
DBP	84-74-2	4.95	+H	C16H22O4	279.15909	80	65.03937	
TBC	77-90-7	7.50	+H	C20H34O8	403.23264	30	129.01824	185.08125
DPNP	131-18-0	8.60	+H	C18H26O4	307.19039	70	149.02337	121.02905
DBS	109-43-3	9.21	+H	C18H34O4	315.25299	20	203.12779	259.18997
DNHP	84-75-3	9.50	+H	C20H30O4	335.22169	35	149.02337	
DNOP	117-84-0	10.15	+H	C24H38O4	391.28429	80	149.02166	
DEHP	117-81-7	10.17	+H	C24H38O4	391.28429	10	149.02332	
DEHA	103-23-1	10.21	+H	C22H42O4	371.31559	10	129.05462	147.06519
DINP	68515-48-0	10.45	+H	C26H42O4	419.31559	35	149.02330	
DIDP	26761-40-0	11.00	+H	C28H46O4	447.34689	35	149.02334	

Table II - Performance characteristics

	LOD (mg/kg)	LOQ (mg/kg)	Trueness %	Repeatability (mg/kg)
BBP, butyl benzyl phthalate	0.06	0.20	102	0.2
DBP, di-n-butyl phthalate	0.05	0.18	109	0.2
DBS, di-n-butyl sebacate	0.79	2.63	80	0.3
DEHA, bis(2-ethylhexyl) adipate	0.28	0.94	100	0.3
DEHP, bis(2-ethylhexyl) phthalate	0.02	0.07	100	0.3
DEP, Diethyl phthalate	0.06	0.19	120	0.2
DIBP, diisobutyl phthalate	0.11	0.35	82	0.2
DIDP, diisodecyl phthalate	0.09	0.32	91	0.6
DINP, diisononyl phthalate	0.03	0.10	100	0.5
DMP, Dimethyl phthalate	0.03	0.10	81	0.2
DNHP, Dihexyl phthalate	0.35	1.17	89	0.2
DNOP, di-n-octyl phthalate	0.28	0.94	105	0.4
DPNP, Di-n-Amyl phthalate	0.10	0.34	79	0.2
DPP, Dipropyl phthalate	0.22	0.73	104	0.2
TBC, tributyl-O-acetylcitrate	0.06	0.21	117	0.2

theoretical value, were considered next to the LOD. Those concentrations were replicated in the oil blank to be spiked. Furthermore, the lowest concentration level for which trueness (see Eurachem Guide, Quick Reference 6) ranged within 70 to 120% was considered for the LOD calculation. According to the above mentioned Eurachem Guide (Quick Reference 2 and 3), since 3 blank samples were also analysed, standard deviation s'_0 was calculated as follows: $s'_0 = s_0 \sqrt{(1/n+1/n_0)}$, where s_0 is the estimated standard deviation of $n = 10$ replicates at or near zero concentration, and n_0 is the number of blank observations (= 3), LOD is equal to $(3 \times s'_0)$.

LOQ was calculated applying a factor $k_q = 10$: $LOQ = 10 \times s'_0$.

Repeatability limit (see Eurachem Guide, 6.6.3 Precision limits) was determined using 10 replicates at concentration level of 1.5 mg/kg, according to the following:

$r = \sqrt{2} \times t \times s_r$, where t is the two-tailed Student t -value for a specified number of degrees of freedom ($\nu = 9$, $t = 1.83$) and at the required level of confidence (in this case, 95%) and s_r is the repeatability standard deviation.

As shown in Table II, LOD ranges from 0.02 mg/kg (DEHP and DMP) to 0.79 mg/kg (DBS), while LOQ ranges from 0.07 mg/kg (DEHP) to 2.63 mg/kg (DBS); trueness ranges from 79% (DPNP) to 120% (DEP) and repeatability from 0.2 mg/kg for most of them to 0.6 mg/kg (DIDP) that are all satisfactory. Furthermore, a linear fitting was tested in the range 1.5 to 6.0 mg/kg ignoring the origin of axis. Table III shows the coefficients of determination (R^2) that are above 0.99 for twelve plasticisers. the lowest one being $R^2 = 0.96$ for DEHP. Thus, a linear regression is a good fit of the real trend of the instrument response over that range of concentrations.

Table III

Linear regression: coefficient of determination, R^2	
BBP, butyl benzyl phthalate	0.9983
DBP, n-butyl phthalate	0.9983
DBS, di-n-butyl sebacate	0.9977
DEHA, bis(2-ethylhexyl) adipate	0.9902
DEHP, bis(2-ethylhexyl) phthalate	0.9649
DEP, Diethyl phthalate	0.9995
DIBP, diisobutyl phthalate	0.9992
DIDP, diisodecyl phthalate	0.9755
DINP, diisononyl phthalate	0.9858
DMP, Dimethyl phthalate	0.9989
DNHP, Dihexyl phthalate	0.9964
DNOP, di-n-octyl phthalate	0.9968
DPNP, Di-n-Amyl phthalate	0.9982
DPP, Dipropyl phthalate	0.9992
TBC, tributyl-O-acetylcitrate	0.9989

3.2 ANALYSIS OF OLIVE OILS FROM THE MARKET

This method was used just as an application to analyse 30 oil samples without any purpose of investigating the olive oils in the European market or comparing different available methods or results as this would be beyond the aims of this paper. Oil samples belonged to the extra virgin, virgin lampante and refined olive oil categories (10 each) taken from the market at production level (oil mills or refineries). As expected, extra virgin oils are normally less contaminated compared to the other categories. Concentrations above calibration ranges were determined after proper dilution.

3.2.1 Extra virgin olive oils

Six plasticisers were found, DEHP (1 sample, 6.3 mg/kg), DIDP (7 samples, 0.6 to 37.8 mg/kg), DINP (5 samples, 2.2 to 28.0 mg/kg), DMP (4 samples, 0.5 to 2.4 mg/kg), DNOP (1 sample, 0.6 mg/kg) and TBC (all samples, 0.3 to 0.6 mg/kg).

3.2.2 Virgin lampante olive oils

Nine plasticisers were found, BBP (3 samples, 0.4 to 2.5 mg/kg), DEHA (3 samples, 2.1 to 3.7 mg/kg), DEHP (all samples, 0.5 to 83.6 mg/kg), DEP (6 samples, 0.1 to 1.0 mg/kg), DIBP (2 samples, 1.1 to 2.2 mg/kg), DIDP (9 samples, 4.0 to 256.2 mg/kg), DINP (8 samples, 4.6 to 54.5 mg/kg), DNOP (5 samples, 0.3 to 0.5 mg/kg), TBC (all samples, 0.3 to 1.3 mg/kg).

3.2.3 Refined olive oils

They showed a dramatic fall of plasticiser content with respect to lampante oils. Only 3 residues were found: DEHP (5 samples, 0.4 to 7.5 mg/kg), DIDP (5 samples, 3.9 to 8.1 mg/kg) e DINP (5 samples, 1.9 to 8.0 mg/kg).

Among the 3 considered olive oil categories, only extra virgin is directly subjected to the mentioned EU Regulation 10/2011 [1] since it fits for human consumption as it is (food). On the contrary, virgin lampante oil does not fit for that purpose and must be refined first, while refined olive oil must be mixed with virgin olive oils to give rise to the olive oil category, suitable for consumption. As mentioned above, Regulation 10/2011 [1] sets limits of migration for a wide range of compounds used in plastic material manufacturing and intended to use for food. Some plasticisers considered in this paper have an SML: they are DBP (0.3 mg/kg), BBP (30 mg/kg), DEHP (1.5 mg/kg) and DEHA (18 mg/kg). Furthermore, some of them belong to numbered Restriction Groups for which that Regulations set total migration limits, SML(T). For these plasticisers, the SML(T) is 60 mg/kg, except for DINP, that is 9 mg/kg.

Among those found in the analysed extra virgin oils, DEHP and DINP were above their SML in just 1 sample out of 10 (6.3 mg/kg and 28.0 mg/kg respectively), while DINP exceeded the SML(T) in that sample only. As to virgin lampante oils, producers do not bother

about contamination because they are intended for refining processes, while refined oils must be controlled as they belong to the edible category of Olive oil, subjected to the mentioned Regulation. Furthermore, it is likely that oil sampling and storage were not performed correctly to preserve them from contamination, e.g., use of not conditioned glass bottles, use of plastic caps etc. Again, paying attention to possible extra contaminations leading to unrealistic values ascribed to oils is extremely important.

4. CONCLUSIONS

The proposed method to determine the amount of 15 plasticisers in olive oils proved to be reliable in terms of repeatability, trueness and response linearity in the range 1.5 to 6.0 mg/kg. LOD and LOQ are comparable to those determined with other methods found in literature on the subject. In the light of these results, matrix effect on instrument calibration can be neglected for routine controls making them easier and cheaper. This method makes use of an HRMS (Orbitrap) detection that may be regarded as too much sophisticated for such a procedure. On the other hand, it was available in our laboratory and we used it, even though we reckon that any other suitable mass detector can be used instead, one its fitness for the purpose has been assessed. As a conclusion, we can state that using this method the extraction and chromatographic separation is carried out quickly with no extra contamination and gives reliable results. This method is one of the easiest, fastest, and low consumables are required for the procedure proving it to be a good choice for high throughput routine analyses in olive oil industry QC Laboratories already equipped with this instrument.

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Author contributions

Both Authors equally shared all contributions to this work

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Declaration of interest

The authors declare they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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