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Iodine deficiency disturbs the metabolic profile and elemental composition of human breast milk

A. Arias-Borrego [a], I. Velasco [b], J.L. Gómez-Ariza [a], T. García-Barrera [a,*]

[a] Research Center on Health and The Environment (RENSMA), Department of Chemistry "Prof. J.C. Vílchez Martín", University of Huelva, Fuerzas Armadas Ave., 21120 Huelva, Spain
[b] Pediatrics, Obstetrics & Gynecology Unit. Hospital de Riotinto, Avda la Esquila 5, 21.660 Minas de Riotinto, Huelva, Spain

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ABSTRACT

Human breast milk (HBM) has a beneficial impact on health programming, growth and neurodevelopment of newborns. Increase in iodine intake is recommended for pregnant women in order to produce enough thyroid hormones to meet foetal requirements. In this work, a combined analytical multiplatform based on gas chromatography coupled to mass spectrometry and ultra-high performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry has been applied in the first metabolomic study of HBM of iodine-deficient women. In addition, the elemental composition of HBM has been determined by inductively coupled plasma triple quadrupole mass spectrometry. Remarkably, 31 metabolites with important biological roles (e.g. glycerophospholipids for neurodevelopment) were seen to be altered in the HBM of iodine-deficient women. The main metabolic pathways altered include lipid metabolism, amino acid cycle, the tricarboxylic acid cycle and glycolysis. Additionally, the concentration of selenium, zinc and copper were seen to be significantly lower in HBM of iodine-deficient women.

1. Introduction

Thyroid hormones are the only compounds that contain iodine with biological activity and, for this reason, this element is essential for mammals. Iodine can only be obtained from the diet, and its requirements increase during pregnancy to fulfil altered maternal thyroid physiology and foetal development (Pearce, Lazarus, Moreno-Reyes, & Zimmermann, 2016). These hormones intervene in many developmental and metabolic processes related to the central nervous system and foetal neurodevelopment (Zimmermann & Köhrle, 2002), affecting the functions of almost all systems. The immune system and the production of antibodies depend on the correct functioning of the thyroid and the presence of adequate levels of thyroid hormones (Reinhardt et al., 1998).

Human breast milk (HBM) is a perfect nutrition source for infants, accurately adjusting its composition to their requirements (Andreas, Kampmann, & Mehring Le-Doare, 2015). Breast milk contains a wide range of nutrients including lipids, complex proteins and oligosaccharides (Thurl et al., 2010).

There are previous studies related to the impact of iodine deficiency on metabolism that pointed out disorders in the basal metabolism of adults, such as carbohydrate metabolism, oxygen consumption and protein synthesis (Mullur, Liu, & Brent, 2014). However, the potential influence of maternal iodine deficiency on HBM composition has not been previously studied. Moreover, it is known that even when the iodine intake is appropriate, other trace elements could affect thyroid metabolism, since they are required for the production, metabolisation and function of thyroid hormones (Köhrle, 2013).

High-throughput approaches for comprehensive metabolomic analysis, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) have appeared as the main analytical techniques in metabolomics. NMR is very reproducible, allows high sample throughput, sample preparation is easy and the availability of databases facilitates the identification of metabolites (García-Barrera, Rodríguez-Moro, Callejón-Leblic, Arias-Borrego, & Gómez-Ariza, 2018). The most powerful advantages of NMR, as traditionally used in metabolomics, are: high sample throughput, no prior separation step requirement, low sample volume consumption, straightforward and fast sample preparation, the sample is not destroyed and it can be applied to *in vivo* measurements (Griffin & Kauppinen, 2007). However, the main drawback is its poor sensitivity, which promotes MS to a powerful alternative for metabolomics. MS also offers high specificity, it can be easily applied to

* Corresponding author.
E-mail address: tamara@dqcm.uhu.es (T. García-Barrera).

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complex samples and allows both qualitative and quantitative analysis (García-Barrera et al., 2018).

In this work, a metabolomic analytical approach has been applied to evaluate the impact of iodine deficiency on the metabolome of HBM, but also to its elemental composition. To this end, iodine deficiency was measured in urine and the metabolome of HBM characterised by a combined analytical platform based on ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) and gas chromatography coupled to mass spectrometry (GC–MS). The elemental composition of HBM was determined by inductively coupled plasma with triple quadrupole mass spectrometry (ICP-QQQ-MS).

2. Materials and methods

2.1. Reagents, solutions and materials

A Milli-Q® Direct 8 system was used for water purification (18.2 MΩ cm; Millipore, Watford, UK). Rhodium used as an internal standard was obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany). Trace-metal grade nitric acid (67–69% purity) and Optima grade hydrogen peroxide (30–35% purity) were obtained from Fisher Scientific (Leicester, UK). Serum Control for Trace Element lyophilised for Trace Elements, Level II was obtained from Clinchek, RECIPE (Munich, Germany) and breast milk NIST 1849 Reference Material (Zn, Se, Mo, Mn, Fe, Cu, Cr) from Sigma-Aldrich (Merck Group, Darmstadt, Germany). Ammonium formate used to prepare the UHPLC mobile phase was obtained from Sigma-Aldrich (Merck Group). LC-MS grade solvents were used for the extraction of metabolites from HBM. Acetonitrile and methanol were purchased from Fisher Scientific (Geel, Belgium), while ethanol, pyridine and formic acid were obtained from Sigma-Aldrich (Merck Group). The derivatising reagents for GC–MS analysis, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), *O*-methoxylamine hydrochloride and TMCS (trimethylchlorosilane), were also purchased from Sigma-Aldrich. Methyl *tert*-butyl ether (MTBE) was obtained from Teknokroma (Barcelona, Spain).

2.2. Instrumentation

A model 8800 Triple Quad ICP-MS (Agilent Technologies, Tokyo, Japan) was used for the determination of elements. Reaction gases used to eliminate interferences were helium and oxygen (high-purity grade, >99.999%) and hydrogen (purity >95%). Most of the analysed elements required 4.5 mL min^{-1} flow-rate of helium. For selenium, a mixture of H_2 (2 mL min^{-1}) and O_2 (40%) was used in MS/MS mode. Isotopes monitored were ^{80}Se, ^{78}Se, ^{66}Zn, ^{64}Zn, ^{65}Cu, ^{63}Cu, ^{57}Fe, ^{55}Mn and ^{53}Cr (dwell time = 0.3 s per isotope).

A tuning aqueous solution of Li, Co, Y and Tl at 1 μg L^{-1} was used to tune the ICP-MS. Skimmer and sampling cones of nickel were used with a sampling depth of 10 mm. A model MARS microwave oven (CEM, Matthews, USA) and MiniXpress polytetrafluoroethylene (PTFE) vessels were used for the mineralisation of HBM samples. Instrumental conditions are summarised in Table S1 (Supplementary Material). For metabolomics, a Trace GC ULTRA gas chromatograph coupled to a model ITQ900 mass spectrometer (Thermo Fisher Scientific) and an Agilent 1290 Infinity UHPLC coupled to a 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Tokyo, Japan) were used. The VF-5 MS Factor Four (30 m × 0.25 mm ID, 0.25 μm film thickness, Agilent Technologies) and C18 Rapid Resolution HD Zorbax Eclipse Plus (2.1 × 50 mm, Agilent Technologies) chromatographic columns were mounted into the GC and UHPLC instruments, respectively. A Speedvac concentrator was used for sample preparation (Thermo Fisher, Waltham, USA).

2.3. Sample collection

Forty transitional HBM samples were obtained by manual expression or by pump within the first 48 h after delivery, and urine samples were taken from pregnant women at their admission for giving birth. Inclusion criteria were: healthy women, single gestation and an absence of perinatal complications or infant pathology. HBM and urine samples were taken during 2015 at the Hospital of Riotinto in Huelva (Spain, Department of Obstetrics and Gynecology). The Local Ethics Committee approved this study, and a written informed consent was obtained from all the women (Code num. 0570-N-20, Regional Ministry of Health and Families of Andalusia). Table S2 shows clinical variables of the women included in this study.

2.4. Determination of iodine in urine

Urinary iodine concentration of the women was measured in the first morning urine by the modified Benotti and Benotti technique (García-Fuentes et al., 2008). Briefly, urine samples were digested with chlorine acid at 110 °C for 90 min and submitted to the Sandelle–Kolthoff reaction, which is the reduction of cerium(IV) to cerium(III) using iodine as catalyst. After the reaction, the absorbance of each sample was measured with a Mini-1240 UV spectrophotometer (Shimadzu Europe, Duisburg, Germany) at 420 nm. The urinary iodine assay is subjected to the programme of the Spanish Association of Neonatal Screening (AECNE) and the Ensuring the Quality of Iodine Procedures (EQUIP) for the external quality assessment of iodine determination in urine. We perform these controls three times/year. The intra- and inter-assay coefficients of variation of iodine in urine were 2% and 4%, respectively. Table S3 shows the urine iodine levels determined in the women under study. According to the World Health Organization (WHO) criteria for pregnant women, those who show urine iodine concentrations below 150 μg L^{-1} are considered iodine-deficient (WHO, 2007).

2.5. Determination of elements in human breast milk

A microwave oven was used for the mineralisation of HBM samples. To this end, an aliquot of 400 μL of each HBM sample was placed into PTFE microwave vessels and weighed. Then, 4 mL of nitric acid and 1 mL of hydrogen peroxide (4:1, v/v) were added. The PTFE vessels were closed after 10 min of pre-mineralisation and introduced into the microwave oven. The power was set at 400 W and a temperature programme was applied from room temperature to 160 °C in 15 min and this temperature held for 20 min. Finally, the elements were determined by ICP-MS using rhodium at 100 μg L^{-1} as internal standard. Three replicates were performed for each sample. For quality control, reference materials and blanks were treated and analysed along with the collected breast milk. Table S1 in the Supplementary Material shows the ICP-MS conditions.

2.6. Metabolomic analysis

A previously published method based on a single-phase extraction protocol was applied for HBM metabolomic profiling (Villaseñor et al., 2014). Briefly, an aliquot of 50 μL of HBM was vortex-mixed with 175 μL of methanol and 175 μL of MTBE. The mixture was vortex-mixed for 1 min, centrifuged at 4000 *g* for 15 min at 15 °C and then analysed by a combined analytical platform based on UHPLC-QTOF (direct injection) and GC–MS (after derivatisation). The derivatisation was carried out according to a previously developed method (Begley et al., 2009) with several modifications. In brief, an aliquot of 150 μL of the extracted metabolites was evaporated to dryness using a Speedvac concentrator. Then, a solution of *O*-methoxyamine hydrochloride in pyridine at 20 mg mL^{-1} (50 μL) was added and mixed vigorously for 5 min in a vortex. Methoxylation was carried out in a water bath at 80 °C for 15 min. Then, MSTFA (50 μL) was added as catalyst and the mixture mixed again for 5 min in a vortex. Silylation was achieved in an oven 80 °C for 15 min. Finally, the supernatants were collected after centrifugation (2039*g* 5 min).

For the chromatographic separation of metabolites using GC–MS, the initial temperature was set to 60 °C, maintained for 1 min and, increased to 325 °C at 10 °C min^{-1}. The injector temperature was set at 280 °C, and a constant carrier gas flow-rate of 1 mL min^{-1} of He was used. The total chromatographic time required for the separation was 30 min. Ionisation of metabolites was performed at 70 eV by electronic impact (EI), and they were detected in an ion trap operated in full-scan mode, screening the range 35–650 *m/z*, with 230 °C as ion source temperature.

For UHPLC-QTOF analysis, the metabolites were separated at a flow-rate of 0.5 mL min^{-1} using 10 mM ammonium formate in water (mobile phase A) and 10 mM ammonium formate in methanol (mobile phase B) using a previously published gradient (Villaseñor et al., 2014): 75% mobile phase B in start, next to 96% B in 23 min, then held until 45 min and next in 100% B for 1 min, and held until 50 min. Finally, starting conditions were restored with an equilibration period of 10 min of 75% B. Samples (1°µL) were injected at 60 °C. During all analyses two reference masses were used: $(M_1\text{-}H)^+ = 121.0509$ ($C_5H_4N_4$) and $(M_2\text{-}H)^+ = 922.0098$ ($C_{18}H_{18}O_6N_3P_3F_{24}$). These masses were infused thorough the system for the correction of masses during data acquisition. The QTOF-MS parameters were as follows: electrospray ionisation mode (ESI), positive and negative polarities, capillary voltages 3500°V (ESI^+) and 4000 V (ESI^-), 1.02 scans/s of scan rate, full-scan mode in the range of 100–1200 *m/z* and 40–1000 *m/z* for MS/MS analysis. For tandem MS analysis, samples were run under the same chromatographic conditions as used in the full-scan analysis. The ions accurately determined by full-scan analysis were submitted to collision induced dissociation (CID) using an isolation width of approximately 1.3 Da. Collision energies (CEs) of 10, 20 and 40 eV were applied to all ions to obtain comparable fragmentation patterns.

2.7. Data processing and identification

The free accessible R platform software (http://www.rproject.org) was used for GC–MS data processing, including peak alignment, feature detection and normalisation. The Thermo File Converter tool allows the conversion of files into CDF format. The signal to noise threshold was fixed at 2, the full width at half-maximum at 3 and the *m/z* range width at 0.1. For peak extraction, the settings used were: S/N threshold = 2, full width at half-maximum (fwhm) = 3 and width for the *m/z* range = 0.1. Then, the alignment of peaks by retention time correction and peaks grouping was performed in three cycles from 5 s to 1 s bandwidth. The locally weighted scatter plot smoothing method (LOESS) was used for normalisation. Partial least-squares discriminant analysis (PLS-DA) was performed using the SIMCA-P software (version 11.5). The values of class separation (R^2) and predictive power (Q^2) were used to assess the quality of the model. Metabolites were annotated by means of the NIST Mass Spectral Library (probability >80%) (version 08) and Kovats retention indices of the metabolites. Some target ions with higher masses and intensities were selected from each metabolite mass spectrum because they are less affected by matrix. In addition, the area qualifier/target ion ratio per metabolite was checked and those with a variation less than 20% chosen (Table S6). After cleaning the background noise, the UHPLC-QTOF data were processed using the Molecular Feature Extraction (MFE) algorithm of MassHunter Qualitative Analysis Software (version B.08.00). This MFE file contained all molecular masses of the mass spectrum obtained from each sample. The Mass Profiler Professional (MPP; version B.10.1) was used to filter and align peaks. The identification of metabolites was performed by metabolite mass matching with online databases such as METLIN (http://metlin.scripps.edu) and HMDB (http://hmdb.ca) with a mass error of 10 ppm. The metabolites that were present in not less than 80% of all samples (quality control and samples) were kept for statistical analysis. PLS-DA of the obtained data were performed using the SIMCA-P software as for the GC–MS data processing.

Univariate analysis (UVA) was performed using the Statistica software, version 8.0 (StatSoft, Tulsa, USA). The normality was tested applying Anderson-Darling (AD) test and then the differences between groups determined with the Student's *t*-test ($p < 0.05$) and then corrected by the Benjamini-Hochberg test.

Metabolites that led to the discrimination of both groups, HBM of iodine deficiency women (IDW) and controls, were considered using the following criteria: a variable importance in the projection of ≥1 (VIP value is a weighted sum of squares of the PLS weight and indicates the impact of the metabolite in the model), *p*-values ≤0.05 and fold-changes included in the interval (+2,−2). Receiver operating characteristic curves (ROCs) were also constructed to evaluate the specificity and sensitivity of altered metabolites considering that metabolites with area under the curve (AUC) values of ≥0.75 are the most important HBM biomarkers of iodine deficiency. The metabolites were identified to MSI Level 2 (putatively annotated compounds based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries) according to the Metabolomics Standards Initiative (MSI; Fiehn et al., 2007). Several metabolites were confirmed with the commercially available standard.

2.8. Quality control of the metabolomic study

For UPLC-QTOF-MS and GC–MS data sets, quality control samples (QCs) were prepared by mixing equal aliquots of each HBM sample. The pool was divided into aliquots that were measured and processed as for any sample. QCs were analysed after every group of five samples and the coefficients of variance (CV) used to evaluate the reliability (Table S4). The applied criterion was that CV% should be <20%.

3. Results and discussion

3.1. Metabolomic profiles of human breast milk of iodine-deficient women obtained by GC–MS and UHPLC-QTOF-MS

As explained previously, metabolites from the HBM were extracted and analysed with a combined analytical platform that consisted of GC–MS and UHPLC-QTOF in positive (ESI^+) and negative (ESI^-) ionisation modes.

A total of 436 individual features were found by ESI^+-UHPLC-QTOF and 104 in ESI^- mode, whereas 210 were obtained with GC–MS. Features were filtered by selecting those that were present in 90% of samples within each group for UHPLC-QTOF (390 features for ESI^+ and 83 for ESI^-) and 85% for GC–MS (170 features).

Fig. 1A and B show the typical UHPLC-QTOF (ESI^+) chromatograms obtained from the HBM of control and IDW, respectively. The UHPLC-QTOF chromatograms obtained by ESI^- from the HBM of control and IDW (Fig. 1C and D, respectively) showed a metabolomic profile with a low number of peaks. Remarkably, the metabolites that eluted from 26 to 46 min presented higher intensities in the HBM of control women (Fig. 1A) than of IDW (Fig. 1B), suggesting a lower content of these metabolites in the HBM due to maternal iodine deficiency. Typical chromatograms obtained by GC–MS can be seen in Fig. 1AS and 1BS, for HBM with normal and deficient levels of iodine, respectively. These last chromatograms need to be statistically analysed to extract information.

The intensities obtained from the *m/z* signals of all the mass spectra (LC and GC analysis) were used for the PLS-DA models to classify the studied groups (HBM of controls and IDW).

Abnormal values or outliers were not observed in the PCA score plots, and the QCs were grouped, indicating good reproducibility and low variability of the measurement. Score plots of PLS-DA obtained from UHPLC-QTOF-MS (ESI^+ and ESI^- modes) and GC–MS allowed a good classification, as can be seen in Fig. 2A, B and C, respectively. Moreover, the parameters of quality confirmed the discrimination power of the models ($R^2 = 0.977$, $Q^2 = 0.812$ for GC–MS; $R^2 = 0.927$, $Q^2 = 0.441$ for ESI^+-UHPLC-QTOF and $R^2 = 0.887$, $Q^2 = 0.321$ for ESI^--UHPLC-QTOF).

Once that the classification of the samples was confirmed, the *m/z* peaks responsible for the grouping of samples were identified by MS/MS

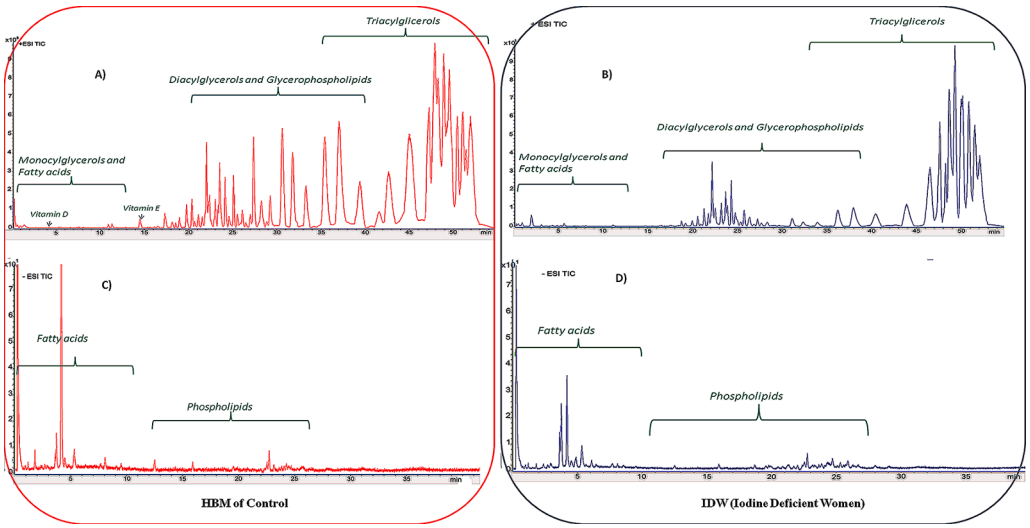

Fig. 1. Total Ion Chromatograms (TIC) obtained from human breast milk in (A) Control and (B) IDW in (ESI^+) mode; (C) Control and (D) IDW in (ESI^-) mode.

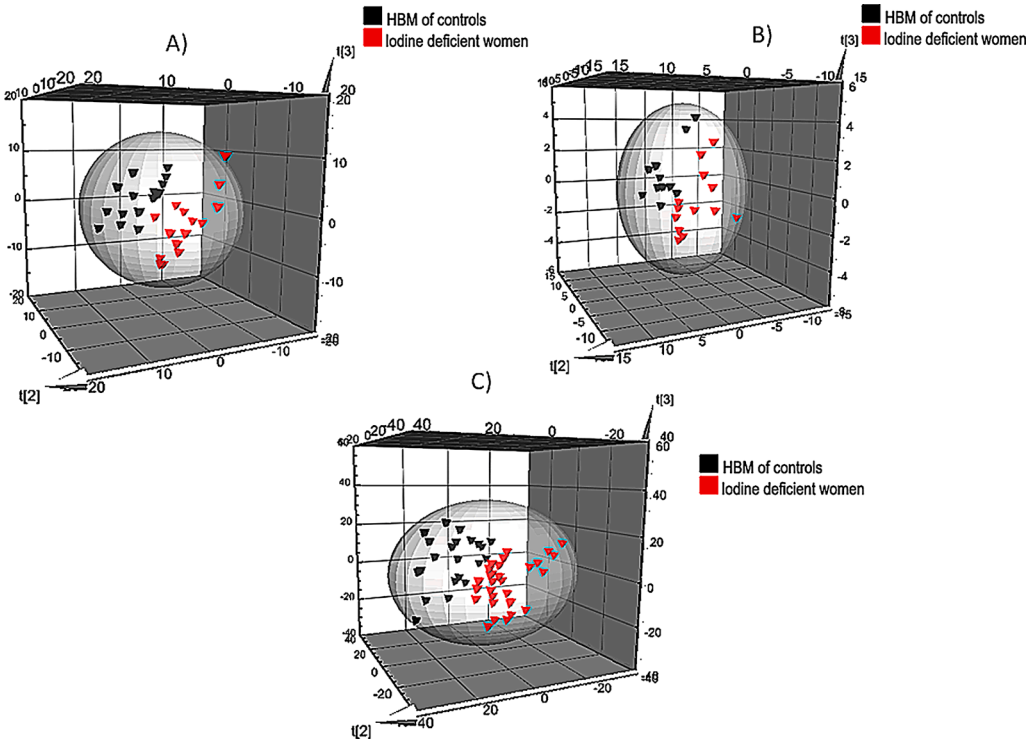

Fig. 2. PLS-DA score plots of human breast milk of iodine deficient women (red dots) and controls (black dots) obtained by: (A) UHPLC-QTOF-MS in (ESI^+) mode; (B) (ESI^-) UHPLC-QTOF-MS in (ESI^-) mode; (C) GC–MS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Table S5), as described in Section 2.7. Tandem mass spectrometry was combined with database searches, including METLIN and HMDB for UHPLC-QTOF mass spectra, while NIST Mass Spectral Library and Kovats retention indices were used for GC–MS mass spectra (Table S6). Typical fragmentation profiles were used to identify compounds in the mass spectra. Table S4 shows the HBM metabolites identified as biomarkers of iodine deficiency in lactating women as well as their fold-changes, accession numbers, *m/z*, p-values and VIP and AUC values. As previously described in Section 2.7, only those signals with VIP > 1, fold-changes between –2 and +2 and *p*-values of ≤ 0.05 were included in Table S4. Metabolites with AUC values of ≥ 0.75 were selected as the most important HBM biomarkers of maternal iodine deficiency. A heatmap diagram showing the differential metabolomic profile of the HMB of IDW against controls is represented in Fig. 3 (using the same statistical criteria as in Table S4). The heatmap shows that 25 metabolites were depressed in the HBM of IDW while 11 metabolites were increased with very good homogeneity among the HBM samples analysed. As can be seen, unsaturated lipids, including several major classes of glycerophospholipids (GPLs) such as phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholine (LPC) and phosphatidylserine (PS), as well as diacylglycerols (DGs) and monoacylglycerols (MGs), were identified in the mass spectra (Table S5, Fig. 3). For example, PC presented typical mass losses of trimethylamine

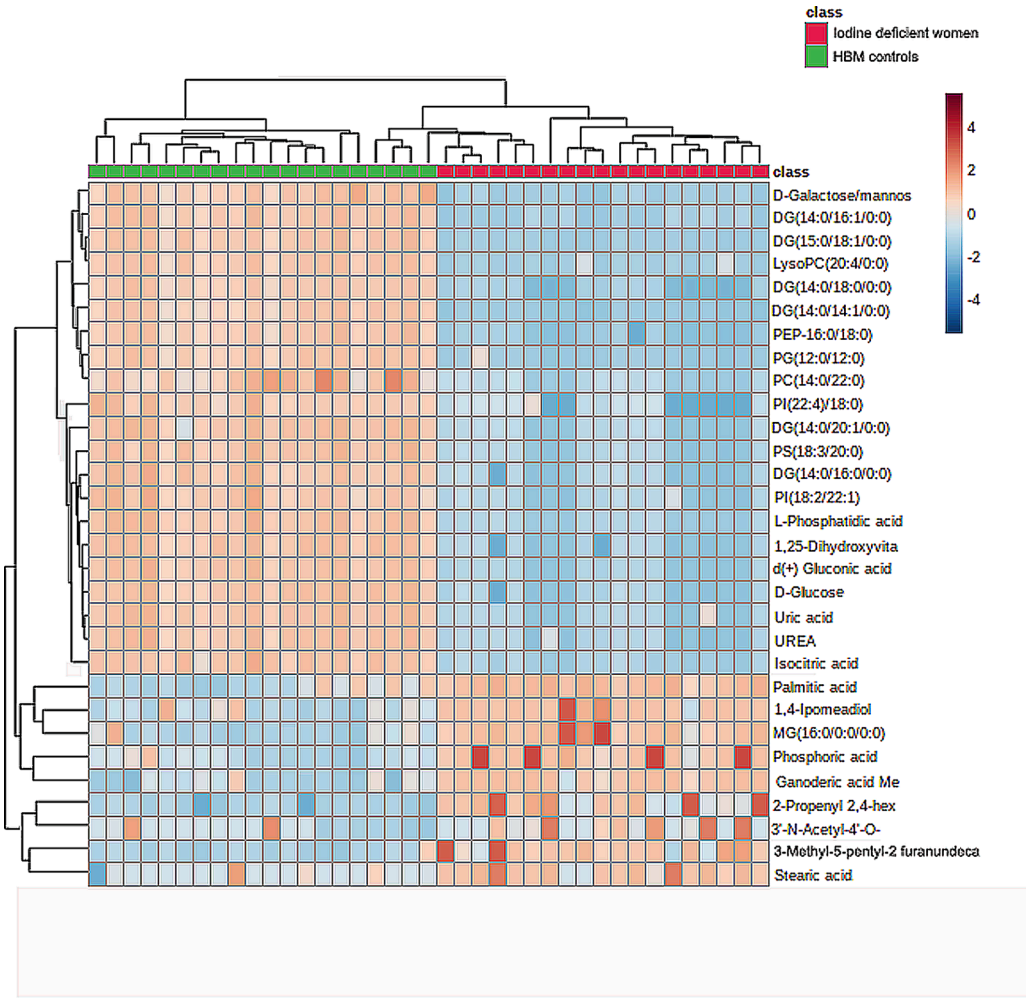

Fig. 3. Heatmap showing the differential metabolomic profile of human breast milk of iodine deficient women and controls.

(*m/z* 60) and phosphocholine (*m/z* 183), as well as the typical ions at 86, 104 and 184 *m/z*. Fragmentation patterns of DGs were identified by the loss of fatty acids to give different ions (A, B, C and D) with typical *m/z* values related to the fatty acid linked to the glycerol chain. Fig. 4 shows the fragmentation profile of PE (P-16:0/18:1) in which the protonated ion is observed at 702.6201 *m/z*, as well as the ions at 141.1752, 239.1682 and 267.1780 *m/z* corresponding to the losses of phosphate head group and the fatty acyl chains of glycerophospholipids that have the patterns of $[RC{=}O]^+$ in ESI(+) mode, respectively.

3.2. *Biological interpretation of the altered metabolites in the HBM of iodine-deficient women*

The Metaboanalyst tool, version 4.0 (https://www.metaboanalyst.

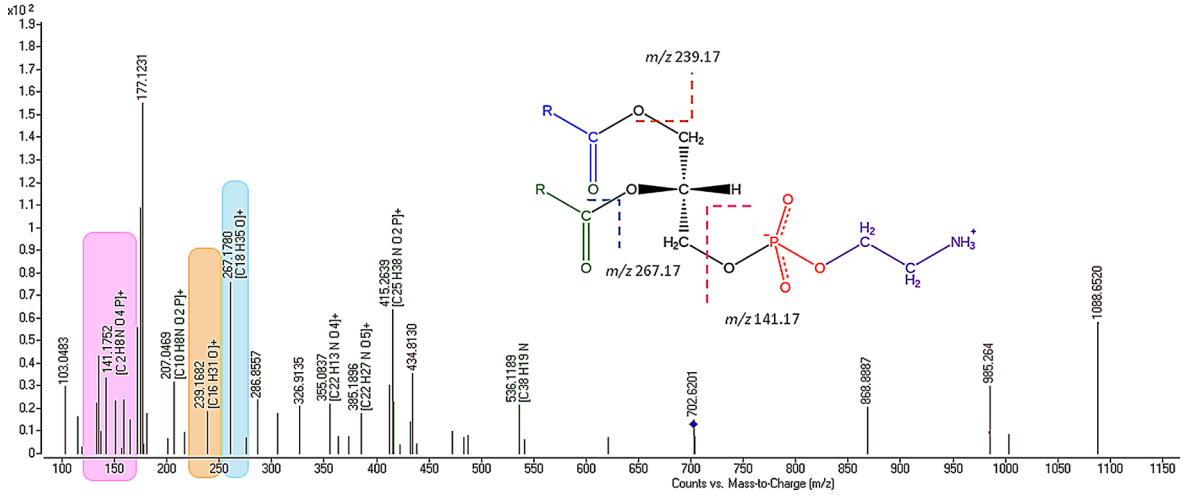

Fig. 4. Fragmentation pattern of the phosphatidylethanolamine plasmalogen PE(P-16:0/18:1) in positive.

ca/) was used to evaluate the possible impact of ID on the different metabolic pathways. Pathway enrichment analysis was performed using the 'Homo sapiens' library with the 'Hypergeometric Test' and the pathway topological analysis with the 'Relative-Betweenness Centrality' algorithms (impact value threshold = 0.1). Fig. 5 shows the resulting metabolic pathways related to altered metabolites in the HBM of IDW. As can be seen, the most altered pathways are lipid metabolism (glycerophospholipid metabolism and biosynthesis of unsaturated fatty acids), the amino acid cycle (arginine biosynthesis), the tricarboxylic acid cycle (TCA, citrate or Kreb's cycle) and glycolysis (glycolysis/gluconeogenesis, galactase metabolism, pentose phosphate pathway, glyoxylate and dicarboxylate metabolism). The lipid metabolism (especially glycerophospholipid metabolism), the citrate cycle and the pentose phosphate pathway present the highest number of altered metabolites in the HBM of IDW (Fig. 5). Otherwise, few metabolites belonging to the arginine biosynthesis pathway are altered in the HBM of IDW, but their significance is the highest. The most altered metabolites in the HBM of IDW and their biological role are discussed in the following sections.

3.2.1. Lipid metabolism

The pathways included in lipid metabolism are the biosynthesis of unsaturated fatty acids and GPL metabolism. HBM contains compounds of pivotal importance for the early postnatal development of the child, such as cortisol, fatty acids (FAs), including polyunsaturated fatty acids (PUFAs) and fragments of the milk fat globule membrane (MFGM). These bioactive compounds are all linked, since cortisol, cortisone and glucocorticoids affect the endocrine and metabolic activities of adipose tissue and energy metabolism (Vicennati et al., 2014). Triglycerides (TGs) account for the highest content of lipids in breast milk (98–99%), but other complex classes such as GPLs and sphingolipids are present (0.2–1.0% of breast milk lipids), located mostly in MFGMs and exosomes (extracellular vesicles) in breast milk.

GPLs are ubiquitous in nature and are key components of the lipid bilayer of cells, as well as being involved in metabolism and signalling (Koivusalo, Haimi, Heikinheimo, Kostiainen, & Somerharju, 2001). There are three major phospholipids (62–80% PLs): PEP, PC and sphingomyelin (SM) and three minor PLs (12–15% PLs): PI, PS and sphingolipid (Giuffrida et al., 2013). All these PLs, except sphingomyelin and shingolipid (all the GPLs), are altered in the HBM of IDW. As can be seen in Fig. 3 and Table S4, the HBM of IDW presents decreased levels of the glycerophospholipids PA(18:0/18:0)L-PA (0.64-fold), PEP(16:0/18:1) (0.56-fold), PC(14:0/22:0) (0.21-fold), LPC(20:4/0:0) (0.62-fold), PS(18:3/20:0) (0.34-fold), PI(18:2/22:1) (0.25-fold), PI(22:4/18:0) (0.27-fold) and PG(12:0/12:0) (0.65-fold). PAs are GPLs in which a phosphate moiety is attached to glycerol and contains one palmitic acid and one oleic acid chain. PAs are not common, but they are key intermediates required for the biosynthesis of PLs and TGs (Athenstaedt & Daum, 1999).

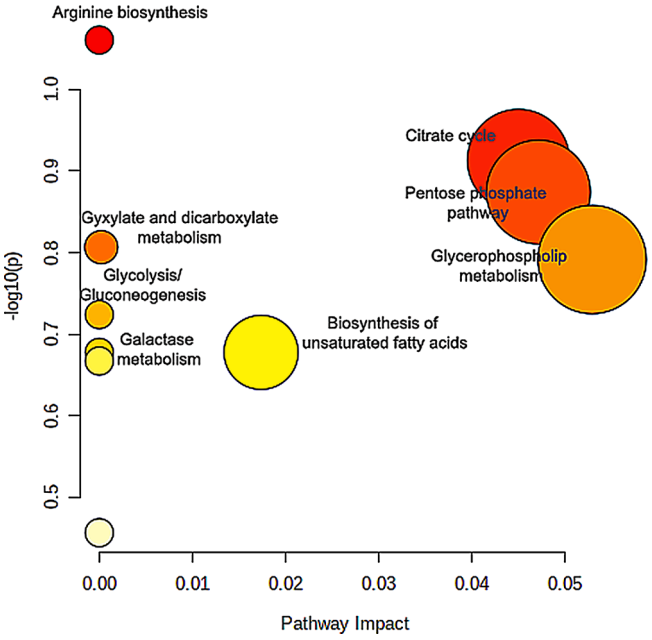

Fig. 5. Summary chart of the interconnected metabolic pathways related to altered metabolites in human breast milk of iodine deficient women. *p*-value is the *p* calculated from the enrichment analysis and the impact is the pathway impact value calculated from pathway topology analysis.

Interestingly, previous works have demonstrated that supplementation with PS and PS/PA complex controls the dysregulation of the hypothalamus–pituitaryadrenal axis (Hellhammer, Vogt, Franz, Freitas, & Rutenberg, 2014), an axis that is affected by thyroid hormones. PEs are GPLs in which phosphorylethanolamine attaches to the *sn*-3 position of diacylglycerol to form PEe. PEs are the second most abundant phospholipids in mammals (Patel & Witt, 2017) and phosphorylethanolamine has been claimed as a constituent of the growth modulator fraction in human milk (Harzer, Franzke, & Bindels, 1984). PCs are GPLs in which a phosphorylcholine is attached to glycerol. These GPLs are essential for choline supply, which is required for neurodevelopment (Verardo, Gómez-Caravaca, Arráez-Román, & Hettinga, 2017). LPCs are monoglycerophospholipids that result from the partial hydrolysis of PCs in which one of the FAs is removed; they supply a small amount of choline to the infant. PS is a GPL in which a phosphorylserine is attached to glycerol. They are carriers for insoluble molecules, improving their intestinal absorption, act as an antioxidants and regulate the cortisol levels (Starks, Starks, Kingsley, Purpura, & Jäger, 2008). Similar to other GPLs, PIs are found in high levels in the dendrites, myelin sheath and synapses, neural structures that are vital for brain connections (Contarini & Povolo, 2013).

Our results demonstrate that the HBM of IDW contains higher levels of palmitic acid (a saturated fatty acid, 1.62-fold), stearic acid (a long-chain fatty acid, 1.80-fold) and other four fatty acyls (Table S4). MG (16:0/0:0/0:0) (1.62-fold) and DG (14:1/14:1/0:0) (1.33-fold) are increased in the HBM of IDW, while five other DGs are decreased. These classes of compounds are related by smell and resemble molecules used by microbes to signal each other.

Remarkably, the HBM of IDW presents 1.35-fold higher levels of the triterpenoid ganoderic acid Me. Triterpenes are immunomodulators that have been proved to develop beneficial roles against inflammation, microbes, viruses and tumoral agents (Ríos, 2010).

3.2.2. Amino acid cycle

Our results demonstrate that arginine biosynthesis is the most significantly altered pathway by maternal iodine deficiency in the metabolome of HBM (Fig. 5). Milk proteins are especially rich in tryptophan (Trp), an amino acid that can be converted into several important molecules (e.g. serotonin, tryptamine, melatonin, niacin, nicotinamide adenine dinucleotide (NAD), phosphorylated NAD, kynureric acid and quinolinic acid, etc). Different Trp-containing peptides found in milk proteins have been shown to inhibit angiotensin converting enzyme (ACE), reducing oxidative stress and showing antidiabetic and satiating-related properties (Nongonierma & Fitzgerald, 2015).

3.2.3. Purine metabolism

Nonprotein compounds such as creatine, uric acid, urea, creatinine and many amino acids account for 25% of the total nitrogen in HBM (Jenness, 1979). IDW present lower amounts of urea and uric acid in their secreted HBM. Although the physiological role of these compounds in the infant can only be speculated upon, it seems that inadequate urea metabolism could be related to sudden infant death syndrome (SIDS; George, 2001).

3.2.4. Glycolysis and the tricarboxylic acid cycle

TCA intermediates provide carbon skeletons for anabolism in the respiratory chain. They have been also recognised as key signalling molecules for cell differentiation and gene expression regulation (Bénit et al., 2014). The third most abundant compounds present in HBM are the oligosaccharides (Coppa et al., 1999). Glycans, usually found as free oligosaccharides or conjugated to lipid or proteins, are a class of bioactive compounds, very abundant in HBM but unable to be digested by the infant; they are especially important for infant health since they are utilised by gut microbiota (Kirmiz, Robinson, Shah, Barile, & Mills, 2018). As can be seen in Table S4, a number of metabolites of the TCA cycle and glycolysis are altered (generally lowered) in the HBM of IDW.

3.2.5. Parathyroid hormone synthesis and endocrine calcium reabsorption

Parathyroid hormone (PTH) is an important modulator of Ca and P homeostasis. Extracellular Ca^{2+} and 1,25-dihydroxyvitamin D3-lactone are the main modulators of PTH secretion. The HBM of IDW presents 1.66-fold higher levels of 1,25-dihydroxyvitamin D3-lactone, which is a seco-cholestane, a hydroxy seco-steroid, a member of the group of D3 vitamins, a secondary alcohol and a steroid hormone. The reason for this increase in the HBM from IDW is unclear and should be investigated further. Otherwise, there is information available about the deficiency levels of vitamin D in HBM, which has been related to musculoskeletal problems, as well as many other diseases (Ioakeim et al., 2017). Vitamin D requirement is increased during pregnancy and lactation, and it has been determined that 6 months of exclusive breastfeeding leads to a 4-fold higher maternal Ca loss than in pregnancy (Ioakeim et al., 2017).

3.3. Elemental composition profile of the HBW of iodine-deficient women

Fig. 6 shows boxplot graphics in which is possible to see the different elemental compositions of the HBM of IDW and controls. Table S7 collects the individual concentrations in each sample, with mean values and standard deviations. Validation parameters obtained by ICP-MS and *p*-values of the ANOVA (HBM of IDW *vs* controls) are indicated in Table S8. As can be seen, when the elemental composition of the HBM of IDW and controls are compared, significant differences are found in the concentrations of selenium ($p = 0.002$), copper ($p = 0.037$) and zinc ($p = 0.002$).

Numerous synergistic and antagonistic interactions have been

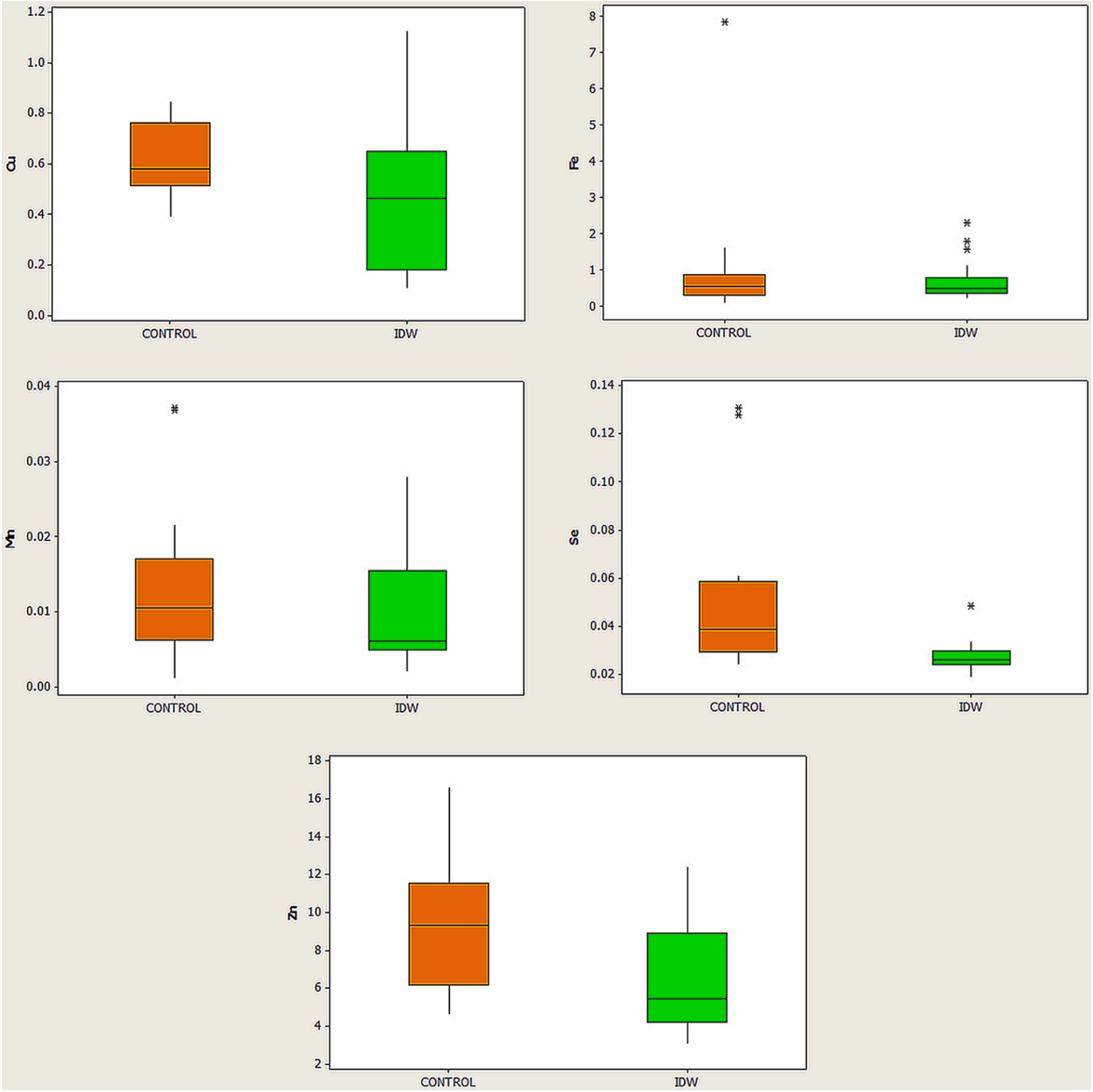

Fig. 6. Boxplots graphics showing the differential elemental composition profile of human breast milk of iodine deficient women and controls.

described between the intake of iodine and other elements, such as selenium or iron. In fact, iodine deficiency could be associated with an excess of selenium intake, as adequate selenium supply ameliorates the adverse effects of iodine excess on the thyroid gland, preventing its inflammation, fibrosis and destruction in animal models (Duntas, 2010; Schomburg & Köhrle, 2008). Thus, an adequate or high intake of a trace element can reveal underlying deficiencies in others, leading to functional impairment or even tissue damage (Köhrle, 2013). Furthermore, during pregnancy, selenium plays an important role in the metabolism of thyroid hormones, which are crucial for foetal development. The thyroid gland contains the highest content of this element per gram of tissue and it is a place where specific selenoproteins are expressed (Köhrle, 2013). The T3 thyroid hormone is the most active in blood, while T4 is the most abundant. Iodothyronine deiodinase (DIO), is a selenoprotein (selenocysteine (SeCys) is incorporated by a specific codon and –SeH is the active centre), which catalyses the deiodination of thyroxine (T4) into triiodothyronine (T3; Schweizer, Weitzel, & Schomburg, 2008). In summary, there are several selenium-containing enzymes involved in thyroid metabolism, such as glutathione peroxidase (GPx), thioredoxin reductase (TRx) and iodothyronine deiodinases (D1, D2 and D3). Selenium is essential for the deiodination of T4 for conversion to T3, but also for removal of excess H_2O_2 via GPx and TRx (Zimmermann & Köhrle, 2002).

Copper also plays an essential role in thyroid function, mainly related to the secretion and absorption of hormones. This element stimulates T4 production and avoids its overabsorption in the blood cells by regulating calcium levels. Moreover, phospholipids, which also influence the stimulation of thyroid hormones, require copper for their synthesis (Arora, 2018). Zinc also plays important roles in the metabolism of thyroid hormones, specifically by controlling the activity of thyrotropin releasing hormone (TRH), deiodination of enzymes and thyroid stimulating hormone (TSH) synthesis. Zinc also shapes the structures of essential transcription factors involved in the synthesis of thyroid hormones. Moreover, previous works have demonstrate that zinc transporters (ZnTs) are present in the pituitary, hypothalamus and thyroid glands, but their role still remains unclear (Severo et al., 2019).

4. Conclusions

A combined analytical platform based on GC–MS and UHPLC-QTOF-MS has been applied to the first metabolomic study of HBM of IDW. In addition, this metabolomic analysis has been combined with the determination of the elemental composition of HBM by ICP-QQQ-MS. Our results demonstrate that the HBM of lactating women with iodine deficiency and normal levels in urine could be clearly distinguished based on their multi-element and metabolomic profiles. Remarkably, 31 metabolites were seen to be altered in the HBM of IDW, these metabolites belonging to different metabolic pathways affecting the mother-offspring transport of important molecules (*e.g.,* glycerophospholipids involved in neurodevelopment). Moreover, the concentration of selenium, zinc and copper was seen to be significantly lower in the HBM of IDW and controls. These results may help further studies aimed at unravelling the candidate bioactive components for the health-promoting effects of HBM.

CRediT authorship contribution statement

A. Arias-Borrego: Conceptualization, Formal analysis, Methodology, Investigation, Validation, Writing – review & editing. **I. Velasco:** Conceptualization, Investigation, Writing – review & editing. **J.L. Gómez-Ariza:** Writing – review & editing. **T. García-Barrera:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131329.

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