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Reference:

Onghena Matthias, Negreira Ferrol Noelia, Van Hoeck Els, Quirynen Laurent, Van Loco Joris, Covaci Adrian.- Quantitative determination of migrating compounds from plastic baby bottles by validated GC-QqQ-MS and LC-QqQ-MS methods
Food analytical methods - ISSN 1936-9751 - 9:9(2016), p. 2600-2612

Full text (Publishers DOI): <http://dx.doi.org/doi:10.1007/S12161-016-0451-4>

To cite this reference: <http://hdl.handle.net/10067/1350130151162165141>

**Quantitative determination of migrating compounds from plastic baby
bottles by validated GC-QqQ-MS and LC-QqQ-MS methods**

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To be submitted to Food Analytical Methods

Abstract

Following the recent prohibition on the use of BPA for the production of polycarbonate (PC) baby bottles in the European Union, baby bottles made of alternative materials, such as polypropylene, polyethersulphone, polyamide, Tritan™ or silicone, have appeared on the market. An initial study done in previous work applying a liquid-liquid extraction (LLE) followed by GC-MS analysis identified a wide variety of migrating compounds. Based on these screening results, the monitoring and quantification of the most important migrating compounds was further pursued. We describe the optimisation, validation and application of a LLE extraction method for the extraction of these migrating compounds adapted to low concentrations. Generally, absolute recoveries between 70 and 110% with RSDs < 20% were obtained. Further, specific and quantitative GC-QqQ-MS and LC-QqQ-MS methods were developed and validated by evaluating the following parameters: sensitivity, selectivity, linearity, accuracy, precision, recoveries and matrix effects. Limits of quantification (LOQ) ranged between 0.6 and 8 $\mu\text{g kg}^{-1}$ for the majority of the selected compounds. Linearity ranged using the Mandel's fitting test between coefficients of determination (R^2) of 0.995 and 0.999. Both repeatability and reproducibility were demonstrated to be satisfactory considering the Horwitz equation. Finally, the methods were applied on a selection of baby bottles representative for the Belgian market. Several compounds previously identified by the screening approach were confirmed and adequately quantified. For instance, azacyclotridecan-2-one had a maximum migration value of 1091 $\mu\text{g kg}^{-1}$; whereas 4-propylbenzaldehyde migrated at 27 $\mu\text{g kg}^{-1}$ and 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB) at 348 $\mu\text{g kg}^{-1}$. However, most of the targeted compounds were not detected or below the LOQ.

44 **Keywords:** Baby bottles; bisphenol-A (BPA) alternatives; migration; GC/LC-QqQ-MS; food
45 contact materials

46

Introduction

Nowadays newborns are, next to breastfeed, mainly fed using a wide range of plastic utensils, such as baby bottles. Until 2011, baby bottles were made mostly of polycarbonate (PC), though the monomer bisphenol-A (BPA) which is used for its production has recently raised concerns regarding its safety as it was reported to exhibit a wide range of toxicological effects such as estrogenic activity (Singh and Li 2012; Vandenberg et al. 2012; Yang et al. 2013; Vandenberg et al. 2013; Mansilha et al. 2013). Since small amounts of BPA could leach from the PC material into foodstuff, many studies have been conducted on BPA release from baby bottles under different conditions (Katrina A. Mountfort, Janet Kelly 1997; Brede et al. 2003; Biedermann-Brem et al. 2008; Ehlert et al. 2008; Maragou et al. 2008; Biedermann-Brem and Grob 2009; Kubwabo et al. 2009; Aschberger and Castello 2010; Santillana et al. 2011). Although the European Union applied a specific migration limit (SML) of 600 mg kg⁻¹ food for BPA (European Commission (EU) 2002), the European Commission recently took the precautionary measure, following the publication of the European Food Safety Authority report (EFSA - European Food Safety Authority 2010), to ban the use of BPA-containing baby bottles (European Union 2011a). In Belgium, more specifically, the Health Council issued an advice in January 2013 (Superior Health Council 2010) regarding the ban on the use of BPA-based polymers in any food contact material intended for children younger than 3 years (Belgian statute book 2012).

Consequently, alternative materials such as polyethersulphone (PES), polypropylene (PP), silicone, polyamide (PA) and a co-polyester called Tritan™ have appeared on the market to substitute the PC baby bottles. Obviously also from these new materials, migration of polymer components, such as monomers, additives (e.g. anti-oxidants, plasticisers), catalysts or even degradation products can be expected (Hoppe et al. 2016). Nevertheless, the migration patterns from these alternative materials have been studied only very briefly

(Simoneau et al. 2011; Simoneau et al. 2012). Only recently a more thorough screening based on a non-target liquid-liquid extraction method (LLE) has been conducted (Onghena et al. 2014; Onghena et al. 2015). This non-target screening approach employed low and high resolution mass spectrometric analyzers coupled to gas (GC) and liquid chromatography (LC) to identify and semi-quantify the most abundant migrating compounds. A wide variety of chemical compounds were determined (e.g. 2,4-di-*tert*-butylphenol, butyl phthalates, 2-butoxyethylacetate, ...), some of which are not authorised by the European Union Regulation No. 10/2011 (European Union 2011b) which defines a Union list of substances that can be used for plastic food contact materials (FCM) together with their SMLs. Compounds which are not listed in the legislation may only be used if the migration levels are confirmed to be non-detectable by an agreed sensitive method. In practice, migrated concentrations in food should remain below 10 $\mu\text{g kg}^{-1}$ (Barlow 2009; Baughan 2015).

Based on the results of this screening, a set of bioassays was applied to each identified compound. A panel of receptor gene assays was used to test the earlier identified chemicals for estrogen, androgen, progesterone, glucocorticoid, thyroid beta, peroxisome proliferator gamma, and aryl hydrocarbon receptor mediated transactivational activity, as these nuclear receptors are involved in numerous regulating processes in human body (Zoeller et al. 2012; Osimitz et al. 2012; Pereira-Fernandes et al. 2013; Contrò et al. 2015). Afterwards, by combining the results of this battery of tests, an overall toxicity scoring was assigned for each compound (Mertens et al. 2016; Simon et al. 2016). Considering these scorings and the detected abundance, a selection was made of the migrating compounds with the highest priority. In order to safeguard any possible risks for public health, it was of the utmost importance to investigate if the specified migration limits of these priority compounds were exceeded in the baby bottle migration samples.

For this purpose, the use of validated analytical methods with a sufficiently low limit

of quantification ((LOQ) - 10 µg kg⁻¹ in simulant) was needed to accurately quantify both the volatile (GC) and less volatile (LC) compounds of concern. Triple quadrupole tandem mass spectrometry (QqQ) is generally the technique of choice for this type of target analysis. To validate the GC- and LC-QqQ methods, the precision (generally accepted as repeatability and reproducibility), the accuracy (often evaluated by repetitively spiking the matrix), and the limit of detection (LOD) were determined as a minimum requirement (European Union 2002; Weitzel et al. 2007).

This work presents the further optimisation of a generic extraction method, as well as the validation of both GC- and LC-QqQ quantitative methods to accurately determine the concentrations of 26 previously selected compounds migrating from baby bottles on the Belgian market. Earlier research conducted on the quantification of toxic compounds migrating from plastic baby bottles mainly focused on only one (e.g. BPA) or a modest number (Simoneau et al. 2011) of target compounds. In other cases, quantification was performed by a semi-quantitative estimation of concentrations when applying a screening approach (Simoneau et al. 2012; Onghena et al. 2014). Therefore, to our knowledge, this is the first time that an accurate quantification by combined GC-MS/MS and LC-MS/MS techniques of a group of migrants selected on the basis of their toxicological profile, from plastic baby bottles was performed.

Materials and methods

Materials

Market Survey and Sampling

Samples were selected based on the market study conducted in our previous research

(Onghena et al. 2014). Briefly, the 24 selected bottles included polypropylene (n = 17), polyethersulphone (n = 2), polyamide (n = 2), Tritan™ (n = 1), silicone (n = 1) and stainless steel materials (n = 1).

Chemicals

Ethanol (absolute for analysis EMSURE®, Reag. Ph Eur.), ethyl acetate (for LC LiChrosolv®), n- hexane (EDC for GC and FID SupraSolv®), sodium chloride (ACS, ISO, Reag. Ph Eur Reag.), sodium sulfate (ACS , ISO, Reag. Ph Eur.), formic acid (for analysis EMSURE® ACS, Reag. Ph Eur), acetonitrile (for LC LiChrosolv®), ammonia (for analysis EMSURE® ACS, Reag. Ph Eur) and ammonium sulphate (extra pure) were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was prepared with Elga Purelab flex system of Veolia Water Solutions & Technologies (Tienen, Belgium). Acetophenone ($\geq 99.0\%$), 4-methylbenzaldehyde ($\geq 97.0\%$), 2-butoxyethoxyethyl acetate ($\geq 99.2\%$), 3,4-dimethylbenzaldehyde (98%), 4-propylbenzaldehyde (97%), 2-undecanone (99%), 2,4,6-trimethylbenzaldehyde (98%), 2,6-di-*tert*-butylbenzoquinone (98%), dicyclopentyl-(dimethoxy)silane (98%), 2,4-di-*tert*-butylphenol (99%), oxacyclotridecan-2-one (98%), 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB, 98.5%), p-*tert*-octylphenol (98.5%), cedrol (purity not specified by the manufacturer), benzophenone ($\geq 99\%$), 2,6-diisopropylnaphthalene (purity not specified by the manufacturer), 2,6-di-*tert*-butyl-4-hydroxybenzaldehyde (98%), azacyclotridecan-2-one (98%), diisobutyl phthalate (99%), dibutyl phthalate (99%), 4-phenylbenzophenone (99%) and methyl oleate ($\geq 99\%$) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The deuterated internal standard (IS) 2,6-di-*tert*-butyl-4-methylphenol-D₂₄ was purchased from Campro Scientific GmbH (Berlin, Germany). Helium (ALPHAGAZ™, 99.999%) and nitrogen (99.99%) were purchased from Air Liquide (Liege, Belgium).

144

145 ***Methods***

146 *Migration testing*

147 Before performing the migration tests, baby bottles were sterilized according to the
148 recommendations by the manufacturer. Therefore, the bottles were filled with boiling water
149 and left during 10 min; this is the only way of sterilizing the bottles that allows collection of
150 the sterilization solution for further analysis. Afterwards, the conventional migration test for
151 “hot fill conditions”, i.e. 2 h at 70°C, was carried out on the baby bottles as prescribed in the
152 EU Regulation 10/2011 (European Union 2011b). The choice of the simulant was also in
153 accordance with the guidelines of the EU Regulation 10/2011 (European Union 2011b). So,
154 H₂O-EtOH (50:50, v/v) was selected as simulant for the migration tests to mimic the use of
155 milk (European Union 2011b). After preheating the simulant on a heating plate to a
156 temperature of 70°C, baby bottles were filled until the specified volume of each bottle. The
157 bottles were then sealed with a plastic cap delivered with the bottle to avoid losses of the
158 simulant by evaporation. Filled bottles were placed in a convection oven for 2 h at a
159 temperature of 70°C. The EU legislation prescribes that when materials, such as baby bottles,
160 are intended to come into repeated contact with foods, the migration test has to be carried out
161 three times using a fresh aliquot of the food simulant for each occasion. After each migration,
162 the simulant was transferred to a glass recipient and stored at +4°C and the bottles were rinsed
163 with ultrapure water for the next migration.

164

165 *Liquid-liquid extraction (LLE) optimisation*

166 Further optimisation of the previously developed LLE method (Onghena et al. 2014)

was necessary for the accurate quantification of GC analysis of the simulant samples considering the relatively low detection levels required ($< 10 \mu\text{g kg}^{-1}$ in simulant). Due to the rather low absolute recoveries for the majority of the selected target compounds and the lack of availability of internal standards for all selected compounds, an increase in these absolute recoveries was aimed. Therefore, several modifications were performed to determine the conditions which produced the best absolute extraction efficiencies for the prioritized migrants. Originally, the LLE was based on an extraction of 30 mL of H_2O -EtOH (50:50, v/v) simulant with a low boiling point organic solvent mixture (ethyl acetate (EtOAc): n-hexane (50:50, v/v)) in order to minimize the loss of volatile compounds during concentration of the sample. This extraction was repeated twice with 10 mL of EtOAc-n-hexane by vortexing this entire mixture one minute. Then, after centrifugation during 5 min the organic extracts were combined in a test tube and evaporated until ± 1 mL was left (method A).

Optimisation of this method was performed with a H_2O -EtOH (50:50, v/v) simulant spiked with the target species at $25 \mu\text{g kg}^{-1}$. In method B, three extractions with 10 mL of EtOAc-n-hexane were carried out. Method C differed from method A by the way of transferring the organic phase, as in this method a large amount of the lower aqueous phase was transferred to the test tube as well. In methods D to G, salts were added to increase the ionic strength of the aqueous phase and so to decrease the water solubility of the analytes. For methods D, E, F and G, respectively 1 g of NaCl and 0.3, 1.5 and 5.0 g of $(\text{NH}_4)_2\text{SO}_4$ were added to the H_2O -EtOH before addition of the organic solvent mixture. For the modified methods, a standard vortex time of 2 min was applied in order to enhance the transfer of the migrants to the organic phase. Also evaporation of the sample was done until ± 5 mL to avoid losses of volatile compounds. In this way, the concentration factor compared to the simulant was 6. After extraction, approximately 100 mg of anhydrous Na_2SO_4 was added and vortexed for 30 sec to dry the organic phase. The method with the best results (Method B) was

afterwards validated and employed for the sample preparation of the migration solutions.

Instrumentation

GC MS-analysis

The EtOAc-n-hexane (1:1) extracts were analysed with an Agilent 7890A gas chromatograph (Agilent JW Scientific, Diegem, Belgium). One μl of extract was injected into a PTV injector in pulsed splitless mode with an injection temperature of 300°C . Separations were carried out in a DB-5ms column (95 % polydimethylsiloxane, $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$) (Agilent JW Scientific). The temperature of the oven was initially set at 60°C for 3 min, and was then increased to 115°C at a rate of $7^{\circ}\text{C min}^{-1}$. Next an increase in temperature of $10^{\circ}\text{C min}^{-1}$ until 240°C followed by another augmentation of $15^{\circ}\text{C min}^{-1}$ until 300°C was applied. Finally, this temperature was maintained during 15 min. The total run time was 42.36 min. Helium was used as a carrier gas; this with a constant flow rate of 1.0 mL min^{-1} during 22 min. Then, the flow was increased to 1.5 mL min^{-1} for 5 min after which it was set back to 1 mL min^{-1} for the remaining analysis time. The GC system was coupled to an Agilent 7000 GC-MS triple quadrupole (QqQ) equipped with an electron impact (EI) ionisation source and operated in multiple reaction monitoring (MRM) mode for the detection and quantification of compounds. The quadrupole and ion source temperatures were set at 150 and 230°C , respectively. N_2 was used as collision gas at a flow of 1.5 mL min^{-1} . The multiplier voltage was 1537 V and 1630 V, respectively for a gain of 10 and 20. In order to improve the number of cycles per second, several acquisition segments were created with different dwell times (15 and 20 ms, respectively). The specific mass spectrometric parameters were tailored for each compound individually in order to monitor the fragmentation ions for each analyte and the internal standard (2,6-di-*tert*-butyl-4-methylphenol- D_{24}) (Table 1).

216 *LC-MS analysis*

217 The migration solutions were directly analysed by LC-MS (without LLE extraction). The
218 liquid chromatographic system was a Waters Acquity Ultra High Pressure LC (UHPLC) fitted
219 with a degasser, a binary high- pressure gradient pump, a thermostated column compartment
220 and an autosampler module. Chromatographic separation was achieved using a Waters
221 Acquity UPLC C₁₈ BEH column (100 mm x 2.1 mm x 1.7 μ m) at a flow rate of 0.4 mL min⁻¹.
222 An injection volume of 10 μ L was applied and during analysis the column was maintained at
223 a constant temperature of 30°C. The mobile phases used for the LC were for the positive
224 ionization mode: H₂O with 0.1 % HCOOH (mobile phase A) and AcN with 0.1% HCOOH
225 (mobile phase B), while for the negative mode H₂O with 0.1 % NH₃ (mobile phase A) and
226 AcN with 0.1% NH₃ (mobile phase B) were used. The following gradient was used for both
227 ionization modes: 0 min: 5% B; 0-6 min: 5-95% B; 6-8 min: 95% B; 8-10 min: 5% B. The LC
228 system was coupled to a Waters XevoTQ-S triple quadrupole mass spectrometer with an
229 electrospray interface (ESI) which was operated in both positive and negative modes for the
230 detection and quantification of the compounds. Source parameters were as follows: a capillary
231 voltage of 3 kV was applied for the ESI + mode and 2.5 kV for the negative mode. Cone
232 Voltage (30 V), cone gas flow (50 L h⁻¹), source temperature (150°C), desolvation gas flow
233 (800 L h⁻¹) and collision gas flow (0.15 L h⁻¹) were the same for both polarities. Two
234 compounds were measured in ESI (+) mode and four in ESI (-) mode. Mass spectrometer
235 parameters were optimized for each compound individually (Table 2).

236

237 **Method optimisation and validation**

238 *Mass spectrometer parameters*

In order to select the specific MRM conditions, individual standard solutions of the selected compounds were injected with the MS spectrometer operating in the scan mode to identify the precursor ion, and in the product ion mode to select the transitions and collision energies (CE). Table 1 (GC-MS/MS parameters) and Table 2 (LC-MS/MS parameters) summarize the retention times, the most intense MRM transitions, and the CE selected for monitoring of the various target analytes. The collision energies were optimized to acquire if possible two (or three) MRM transitions (at least one quantifier and if possible one or two qualifiers) for each compound and for the internal standard. The most abundant transition in terms of signal to noise ratio (S/N) was chosen as quantifier (Q) and the second most abundant transition as qualifier (q). The MRM1/MRM2 ratio was monitored for variation (relative standard deviation (RSD <30%)) to provide an additional identification criterion besides the retention time (RSD <5%).

Method validation

The performance of the method was evaluated by an in-house validation of the method. The following characteristics were assessed: precision, accuracy, selectivity-specificity, linearity, calibration range, recovery, matrix effects, lower limit of quantification (LOQ) and sensitivity.

For the GC-method, multi-component calibration curves (n=3) based on an internal standard (IS) with ten calibration points were made. Carryover was evaluated by injecting a blank sample fortified with internal standard after the highest concentrated (1000 $\mu\text{g kg}^{-1}$) calibration standard injection in the instrumental sequence.

For each compound, a calibration curve was established with R^2 according to the linear model, which was examined by the Mandel's fitting test (Mandel 1964). The precision in the form of repeatability and intermediate precision (reproducibility) were examined based on the Horwitz equation for an expected low (10 $\mu\text{g kg}^{-1}$), intermediate (50 $\mu\text{g kg}^{-1}$) and high (150

$\mu\text{g kg}^{-1}$) control concentration. For repeatability, five replicates of the control sample were analysed by the same person on the same day. For reproducibility, the control sample was analysed by the same person in the same conditions, with five replicates per day for three different days. The recoveries were calculated following the method B. LOQ and limit of detection (LOD) were calculated for signal to noise ratio (S/N) 10 and 3, respectively, based on five replicates. In addition, a zero extract sample (processed matrix sample without analyte with IS) and a quality control (QC) sample at an intermediate concentration in the expected sample concentration range ($50 \mu\text{g kg}^{-1}$) were included. Matrix effects were evaluated and quantified during method optimisation based on a blank simulant sample. Specificity and selectivity were checked based on retention times and MRM ratios (MRM1/MRM2).

The LC-method was validated based on the same principles, though some minor practical changes were adapted here, as simulant samples were injected directly into the LC-MS/MS without any pre-treatment. Calibration curves were prepared in the same medium as the samples ($\text{H}_2\text{O-EtOH}$ (50:50, v/v)) with seven calibration points ranging from 1 to $100 \mu\text{g kg}^{-1}$ and thus no matrix effects were studied. A blank sample consisted in blank simulant without any treatment, while simulant spiked at $50 \mu\text{g kg}^{-1}$ served as a QC sample. Linearity was also evaluated with the Mandel's fitting test, and precision and accuracy were determined again based on the Horwitz equation. The concentrations used here (low ($5 \mu\text{g kg}^{-1}$), intermediate ($10 \mu\text{g kg}^{-1}$) and high ($75 \mu\text{g kg}^{-1}$) were different considering that the expected concentrations were lower than for the GC method since no concentration step was included.

Results and Discussion

LLE method development

Optimisation of extraction conditions

The results of the absolute recoveries of the previously developed LLE method (method A) showed low values for the most of the selected compounds within the first and second extraction step (generally between 20 and 50%) (Table 3). A third extraction step was therefore added in method B in order to obtain quantitative recoveries. Although the labour intensity of the method was significantly higher adding this extra extraction step, higher recoveries were obtained (generally between 70 and 110%), yet azacyclotridecan-2-one remained at low values even after 3 extraction steps (Table 4). For method C, the transferred extract still contained an important amount of water and precaution had to be taken when pipetting 200 μ L of concentrated extract to a vial for GC injection, since water should not be present to avoid damage of the GC column. For this adaptation, a slight increase in the absolute recoveries was observed, although they were still lower than for method B (Table 4).

The addition of NaCl to the H₂O-EtOH mixture before extraction showed an improve of the extraction response for some analytes (e.g. 2,4,6-trimethylbenzaldehyde; 2-butoxyethyl acetate). For some compounds, the addition of salt played an opposite effect as recoveries even became lower than before (e.g. 2,4-di-*tert*-butylphenol) or splitting of the chromatographic peak occurred (not accurately quantifiable anymore, e.g. benzophenone) which made this addition not appropriate. The influence of (NH₄)₂SO₄ was first tested for the addition of 0.3 g, demonstrating only a slight increase in the recoveries of most compounds. When adding 1.5 and 5 g of (NH₄)₂SO₄, a 3-phase system was formed. Both methods were therefore discarded.

When comparing the tendency of the recovery values and repeatability of the target compounds between the different methods, it was clearly that method B exhibited the most significant performance enhancement. This method was therefore validated and afterwards applied to real baby bottle samples. All detailed validation parameters were shown in Table 4.

Method validation

At first, for the GC-analysis, matrix effects for the proposed method B were assessed with extracts of blank simulant samples spiked at $125 \mu\text{g kg}^{-1}$ ($n=5$) after LLE by comparing responses of the target analytes (after subtracting the peak areas corresponding to the native analytes present in the sample) with those measured in blank simulant samples spiked before extraction. Since no matrix effects were present, an external calibration against standards prepared in EtOAc:n-hexane (1:1) was used to measure the levels of those compounds in simulant samples.

The linearity of the method was investigated with standards prepared in EtOAc:n-hexane (1:1) at ten different concentrations, from $1 \mu\text{g kg}^{-1}$ (or the limit of quantification if higher) to $1000 \mu\text{g kg}^{-1}$ (0.7; 3.5; 7; 17.5; 35; 70; 175; 350; 700 and $1000 \mu\text{g kg}^{-1}$). The concentration of the IS was in all cases $300 \mu\text{g kg}^{-1}$. The calibration curves were obtained by plotting the peak areas of the analyte and the internal standard versus the spiked concentrations and were not weighted.. An “F value” was calculated for each substance. The obtained “F values” were compared with a tabulated F value, corresponding to the F-distribution with 1 and $n-3$ degrees of freedom and a probability of 99%. The $F=0.99$ was 16.26 for all compounds. According to the Mandel’s fitting test, the straight line regression model is preferred when the calculated “F value” is below F 0.99. All compounds gave a linear response in the above described calibration range ($TV < F$), with determination coefficients (R^2) higher than 0.990, except for 2,6-di-*tert*-butylbenzoquinone, *p-tert*-octylphenol, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, azacyclotridecan-2-one and 4-phenylbenzophenone with R^2 values between 0.977 and 0.984 (Table 4). The instrumental limits of detection (LODs) and quantification (LOQs) were experimentally estimated from the lowest level included in the calibration curve as the concentration of analyte giving a S/N of 3 and 10, respectively. Instrumental LODs varied between 0.2 and $10.3 \mu\text{g kg}^{-1}$, whereas the

LOQs ($S/N = 10$) ranged between 0.7 and $34.2 \mu\text{g kg}^{-1}$.

The precision of the method was evaluated with simulant spiked at three different concentrations: 10, 50 and $150 \mu\text{g kg}^{-1}$ extracted within the same day (repeatability) and on different days (reproducibility) ($n=5$). The repeatability (r) and the intra-laboratory reproducibility (R_w) were evaluated by calculating the relative standard deviations (RSD) for these two parameters (RSD_r and RSD_{Rw}) according to the ISO-5725-2 guidelines (Interscience publications 1994). These RSD's are compared to the RSD's obtained from the Horwitz equation (Horwitz et al. 1980; Horwitz and Wood 2000) and modified by Thompson (Thompson 2000). The calculations are carried out for each concentration level of the validation and the results are given in Table 4. When the obtained RSDs are significantly below the RSDs derived from the Horwitz equation, the method showed good precision at the levels of interest for the compound. However, some compounds exhibited already a lower repeatability when applying the previously optimized extraction (e.g. *p-tert*-octylphenol; 4-phenylbenzophenone; azacyclotridecan-2-one) and were therefore included in the LC-MS analysis method.

Reproducibility was evaluated for $n=5$ extractions processed for 3 consecutive days (Table 4). Compounds which previously exhibited a poor repeatability showed the same pattern here and generally did not suffice the tolerance level calculated from the Horwitz equation for reproducibility (22, 22, and 21% for the different concentrations). 2,6-Di-*tert*-butyl-benzoquinone also presented higher RSDs values than allowed with 31 and 26% for the 10 and $50 \mu\text{g kg}^{-1}$ concentration respectively, together with benzaldehyde, 2,4,6-trimethyl (25% for $50 \mu\text{g kg}^{-1}$) and acetophenone (24% for $50 \mu\text{g kg}^{-1}$). Since this is a multi-residue method, it was therefore not possible to optimize all parameters equally well for different compounds and sometimes compromises had to be made. 2,6-diisopropylnaphthalene (DIPN) gave poor results for both repeatability and reproducibility due to blank contamination.

Procedural blanks, performed with simulant without fortification, showed the absence of significant contamination problems for most compounds. However, both DIPN and bis(2-ethylhexyl)phthalate (DEHP) were systematically detected in blanks extracts at varying concentrations and were therefore quantified only semi-quantitatively.

The achieved method LOQs ranged from 0.6 to 8.0 $\mu\text{g kg}^{-1}$ simulant, with the exceptions of cedrol (9.7 $\mu\text{g kg}^{-1}$) and azacyclotridecan-2-one (9.6 $\mu\text{g kg}^{-1}$) which were just around the proposed “non-detection limit” of 10 $\mu\text{g kg}^{-1}$. Methyl oleate exhibited a high LOQ (25 $\mu\text{g kg}^{-1}$) due to its low sensitivity. For DIPN (13.4 $\mu\text{g kg}^{-1}$), the high LOQ was of course observed due to its presence in the procedural blanks. The same issue was also faced for DEHP, a common contaminant present in almost any plastic material used for laboratory work (Nguyen et al. 2008). For both compounds, rather high RSDs (>25%) were obtained. Since DIPN and DEHP were not detected in the previous screening method (Onghena et al. 2014), we still decided to include them in our method considering their endocrine activity in different bioassays (Simon et al. 2016) and public concern. When analytes were present in the procedural blanks, the mean of the concentrations in those blanks was used. If the latter value was lower than 3 x SD, this was adopted as LOQ.

Absolute recoveries were calculated by comparing the analyte peak area obtained in spiked samples (after subtracting the amount of the analyte in the blank ($n = 5$), if present) and in standard solutions with equivalent concentrations. Relative recoveries were calculated by comparing the absolute recoveries obtained for each analyte and the internal standard. Relative recoveries calculated with respect to the IS were between $65 \pm 2\%$ and $117 \pm 5\%$ for the different concentration levels.

Finally, for positive confirmation of the presence of a compound in a sample, the GC retention time of the compound in the sample had to match that of the standard with a margin of $\pm 5\%$, and its precursor-product ion ratio could not deviate more than 30% (depending on

the MRM1/MRM2 value) from the ratio in the standard (European Union 2002).

For the LC-MS validation, linearity was investigated with standards prepared in H₂O-EtOH at eight different concentrations (1, 2, 5, 10, 25, 50, 75 and 100 µg kg⁻¹). The Mandel's fitting test demonstrated also here a linear response in the proposed calibration range with R² for all compounds between 0.995 and 0.999. For p-*tert*-octylphenol and 4-n-nonylphenol only one MRM transition was selected (quantifier). The LOQ was determined at 1 µg kg⁻¹ for all components. Repeatability and reproducibility were tested at three levels (5, 10 and 75 µg kg⁻¹) and all RSDr and RSDRw resulted below the required Horwitz values (Table 5) consequently demonstrating the adequate measurement of those compounds that resulted to be troublesome with GC-MS analysis. The ratio of the concentrations obtained and the theoretical concentration for the 10 µg kg⁻¹ calibration level gave us recoveries between 98 and 110%.

Migration from baby bottles

The experiments to test the migration from the selected baby bottles were all carried out following the EU legislation which defines a Union list of substances that can be used in plastic food contact materials together with their SMLs (European Union 2011b). The substances not included in the Union list can be used if they are not classified as carcinogenic, mutagenic or reprotoxic (CMR) and if they are not detectable in the food with an appropriate sensitive method. In practice, the concentrations of these substances in food should remain below 10 µg kg⁻¹ (Barlow 2009).

Furthermore, the Regulation also defines the use of simulants and the testing conditions to be implied. For baby bottles, a H₂O-EtOH (50:50, v/v) solution was therefore used as simulant for milk and three consecutive migrations were performed during 2h at 70°C. These consecutive migrations are prescribed by the Regulation to mimic the repetitive use of a FCM. To assess its final compliance, the concentrations of migrating compounds

measured in the third migration step have to be compliant with those specified in the Regulation. On the one hand, the previously optimized and validated extraction method was applied to detect and quantify the migration from baby bottles made of plastic alternatives to PC for the more volatile compounds. On the other hand, for the more polar migrants that needed to be analysed with LC-MS, direct injection of the H₂O-EtOH (50:50, v/v) simulant samples was possible.

Polypropylene (PP) baby bottles

PP resulted to be the most used plastic as an alternative to PC baby bottles on the Belgian market, representing more than 60% of the market share. Previous studies already demonstrated that a wide variety of chemicals present in PP plastics could migrate towards the food (McDonald et al. 2008; Alin and Hakkarainen 2010; Chang et al. 2016). For baby bottles, severe differences in the identity and the concentration of the compounds migrating from the different PP bottles were observed (Simoneau et al. 2012; Onghena et al. 2014) which agreed with the data obtained in this study. Indeed, when quantifying those compounds that were previously selected to be of priority interest, some bottles exhibited relatively large concentrations of migrants that in other bottles were not even seen. An overview of the results is given in Table 6. Bottles number 5, 7 and 25 in particular exhibited a wide variety of migrants at relatively high concentrations (e.g. 2-butoxyethylacetate in n° 25: 946 µg kg⁻¹) whereas from other bottles such as 9, 26 or 27, only 1 or 2 compounds migrated at concentrations just above the LOQ. Generally only 2 compounds with a specified SML were detected, benzophenone and dibutyl phthalate. Benzophenone was measured above the LOQ in only 3 out of 17 PP bottles, though in one case it was found up to 97 µg kg⁻¹ (bottle 12). Yet, for this specific compound the detected migrating concentrations remained far below the SML of 600 µg kg⁻¹. Dibutyl phthalate (SML= 300 µg kg⁻¹) was identified in one bottle at

very low concentration ($5 \mu\text{g kg}^{-1}$). None of the other targeted compounds detected in PP bottles were listed in the EU Regulation as authorised substances. Some of them migrated at concentrations significantly higher than $10 \mu\text{g kg}^{-1}$ (e.g. dicyclopentyl(dimethoxy)silane: $117 \mu\text{g kg}^{-1}$ in bottle 8) and therefore their origin and corresponding toxicological profile should be further investigated. 2,4-di-*tert*-butylphenol was detected in >90% of PP bottles though only for 4 specific bottles the concentrations exceeded the LOQ varying from 12 to $118 \mu\text{g kg}^{-1}$. LC-QqQ analyses only revealed the presence of p-*tert*-octylphenol ($7 \mu\text{g kg}^{-1}$ in bottle 7) and 4-phenylbenzophenone ($7 \mu\text{g kg}^{-1}$ in bottle 21).

Baby bottles made of other materials

Other baby bottle materials offered on the Belgian market were PES, PA, Tritan™, silicones and stainless steel. In the PES bottles, none of the selected compounds except for acetophenone (at very low concentration: $3 \mu\text{g kg}^{-1}$) were encountered. It was interesting to take into account though that BPS, a building block for PES with similar endocrine disrupting properties (EDC) to BPA (Kuruto-Niwa et al. 2005), was not detected in the samples. These results were consistent with an earlier study (Simoneau et al. 2011).

PA bottles exhibited just like PES a very low variety of migrating compounds, though the PA monomer azacyclotridecan-2-one was detected at relatively high concentrations (924 and $1091 \mu\text{g kg}^{-1}$). Yet, this was still far below the SML of $5000 \mu\text{g kg}^{-1}$ of this compound.

The Tritan™ bottle showed, in contrast to both PES and PA, a rather wide variety of migrants. Nevertheless the concentrations detected in the third migration step were low (e.g. dicyclodipentyl(dimethoxy)silane: $10 \mu\text{g kg}^{-1}$; 2,4-di-*tert*-butylphenol: $8 \mu\text{g kg}^{-1}$) and only for 4-propylbenzaldehyde ($27 \mu\text{g kg}^{-1}$) the concentration was higher than $10 \mu\text{g kg}^{-1}$.

The migration profile of the silicone baby bottle found on the Belgian market was also tested. Since EU Regulation 10/2011 is only applicable for plastics, it cannot be used for

silicones. However, a resolution of the Council of Europe that contains inventory lists of substances is available and can be used for the interpretation of the data observed in this study (Council of Europe 2004). Previous research (Simoneau et al. 2012; Onghena et al. 2014) indicated already the migration of possible EDCs. Indeed, several components with a potential endocrine activity such as phthalates were identified (diisobutyl phthalate: $15 \mu\text{g kg}^{-1}$; dibutyl phthalate: $11 \mu\text{g kg}^{-1}$). Furthermore 3,4-dimethylbenzaldehyde was encountered at $15 \mu\text{g kg}^{-1}$ and 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB) at a concentration of more than $348 \mu\text{g kg}^{-1}$. This substance was authorised by European Union legislation but may only migrate at a level of $5000 \mu\text{g kg}^{-1}$ from single-use gloves according to EU Regulation 10/2011. 2,6-Di-*tert*-butylbenzoquinone ($8 \mu\text{g kg}^{-1}$) and benzophenone ($9 \mu\text{g kg}^{-1}$) were both detected below $10 \mu\text{g kg}^{-1}$. For the stainless steel bottle none of the targeted compounds could be detected.

Conclusions

A LLE method was optimized for the extraction of chemicals migrating from plastic baby bottles and which were identified as of toxicological interest. GC- and LC-MS/MS analysis methods were successfully validated to accurately determine the concentrations of these compounds in the selected food simulant. The analysis of the migration solutions confirmed the presence of previously detected compounds and they were adequately quantified. Compounds with an SML which were regulated by the EU Regulation No. 10/2011 did not exceed these specified values. Yet, other migrants which were not specified in the EU 10/2011 positive list with authorised materials were detected sometimes above $10 \mu\text{g kg}^{-1}$. Therefore, further research is needed to understand the potential health risks associated with the migration of these quantified compounds. Finally, Bisphenol-A and

487 Bisphenol-S were not detected in any of the migration solutions, indicating that the new
488 plastics used as the replacement of polycarbonate are not leaching these bisphenols.

489

490 **Compliance with ethical standards**

491 **Funding**

492 Matthias Onghena thanks the Federal Government Service for Public Health of
493 Belgium for funding [grant number ALTPOLYCARB (RT 12/10)]. Noelia Negreira
494 acknowledges the University of Antwerp for her postdoctoral fellowship.

495 **Conflict of interest**

496 Matthias Onghena declares that he has no conflict of interest. Noelia Negreira declares
497 that she has no conflict of interest. Els Van Hoeck declares that she has no conflict of interest.
498 Laurent Quirynen declares that he has no conflict of interest. Joris Van Loco declares that he
499 has no conflict of interest. Adrian Covaci declares that he has no conflict of interest.

500 **Ethical approval**

501 This article does not contain any studies with human participants or animals performed
502 by any of the authors.

503 **Informed consent**

504 Not applicable.

505

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