An improved and miniaturized μ-QuEChERS strategy for isolation of polyphenols for quality control of baby foods

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Microchemical Journal 139 (2018) 110–118

https://doi.org/10.1016/j.microc.2018.02.026

ABSTRACT

An improved and miniaturized quick, easy, cheap, effective, rugged and safe (μ-QuEChERS) strategy combined with ultra-high pressure liquid chromatography coupled to a photodiode array detection system (UHPLC-PDA) was developed and optimized for the determination of twelve polyphenols in different baby foods samples. The proposed analytical approach proved to be highly cost-effective and environmentally friendly, since it showed good extraction efficiency using few amounts of sample (0.3 g), organic solvents (1000 μL), clean-up sorbents (87.5 mg) and partitioning salts (0.2 g), producing minimal waste disposal and reducing analysis time. Method performance was validated in terms of selectivity, linearity, limits of detection and quantification, matrix effects, precision and accuracy using different baby food matrices according to their composition. The overall average recoveries ranged from 71-100% with relative standard deviations lower than 6% (n=18). The method was successfully applied to commercial baby food samples (including pureed solids, juices and porridges), and their total phenolic content (TPC) and antioxidant capacity (RSA) were also investigated. Results allowed characterizing the abundance of the selected polyphenols in the samples, and the highest RSA and TPC were found in the fruit-based baby foods. Thus, this work represents a first approach to determine the nutritional quality of these products.

Keywords: μ-QuEChERS, Polyphenols, Baby foods, UHPLC-PDA, Antioxidant capacity, Food analysis

1. Introduction

The first year of life of babies is a very sensitive and critical period for the optimal growth and correct development of their nervous, digestive, reproductive, respiratory and immune systems, therefore, the composition of their diet plays an important role to ensure good nutrition and prevent possible future diseases [1]. Nowadays, an increasing number of families feed their babies with commercial infant formulas or solids such as fruits, vegetables and meat or fish pureed baby foods, what has led to the availability of a wide variety of these products on pharmacies and supermarkets. In the food field, research related to baby foods has mainly focused in the development of analytical methods that ensure food safety of these products, while their composition and nutritive quality have often been underestimated. In this sense, research regarding the content of phenolic compounds and antioxidant activity of baby foods is very limited [2, 3]. The nutritive value of these products depends on the raw materials that are used for their production; therefore, the ones with fruits and vegetables in their composition can be an excellent source of polyphenols and other antioxidants compounds. However, food processing effects must be also considered, since homogenizing or heating the raw materials may result in modifications of the initial levels and properties of these compounds. Polyphenols are secondary metabolites of plants which are considered bioactive compounds since many studies have reported multiple beneficial effects related to their consumption, such as anti-carcinogenic, anti-atherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic effects [4, 5]. Moreover, there are evidences that diets rich in polyphenols support a role in the prevention of future diseases such as cancer, cardiovascular diseases, diabetes, neurodegenerative diseases and osteoporosis [6]. These positive effects on the human organism highly depend on their form, whether they are present as glycosides or in their aglycone form, since their metabolism depends on their solubility and stability. So it is not only important to know in what amount these compounds

can be present in foodstuffs, but also to know in what form they can be found and how the technological food processes may affected them.

Evidences on the health benefits of polyphenols and their influence on food quality have promoted the development of analytical strategies for their identification and quantification. However, the analysis of polyphenols in food samples is relatively complex due to the great variety of compounds that can be present, with different polarity, size and form (glycosylated or in their aglycone form), but also because many of these compounds occur at low concentration levels in foodstuffs and can be subjected to matrix interferences. Therefore, an appropriate sample pre-treatment method is crucial in the analytical process. In recent years, there has been a trend to develop new extraction and clean-up approaches to simplify sample preparation, moving towards more environmentally friendly techniques and more efficient analysis [7]. In this sense, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is an extraction and clean-up technique originally developed in 2003 by Anastassiades et al. [8], as a "green" user-friendly and cheap approach for the multiresidue analysis of pesticides in fruits and vegetables. This approach includes a initial single-phase extraction with acetonitrile followed by salting-out extraction/partitioning with $MgSO_4$ and NaCl, and finally clean-up using dispersive-solid-phase extraction (*d*SPE). Nowadays, due to its high flexibility, the QuEChERS concept has spread beyond its original field of application to be adapted to other analytes and food matrices, thus it has been used for the determination of acrylamide [9], veterinary drug residues [10], alkaloids [11] and mycotoxins [12], among others.

Recently, QuEChERS has been successfully applied for multiresidue analysis of polyphenols in vegetable samples [13], and since it has been previously reported elsewhere that the original method can be miniaturized [14], the aim of this work was to developed an improved cost-effective μ-QuEChERS extraction technique which produced minimal amount of waste disposal in comparison to the classical extraction procedure for the determination of

polyphenols in different baby foods, since these compounds have been poorly studied in these matrices. Important parameters which might affect extraction efficiency, such as partitioning solvents and sorbents used in the clean-up step, were investigated and optimized. As far as we know, this is the first time this technique is evaluated and applied for the analysis of polyphenols in baby food samples. Moreover, the total polyphenolic content (TPC) and antioxidant capacity of all the samples were investigated using the Folin-Ciocalteu's colorimetric method and the DPPH$^{\bullet}$ free radical-scavenging activity (RSA), respectively. Thus, this work represents a first approach to determine and evaluate the nutritional quality of this kind of products.

2. Materials and Methods

2.1. Reagents, materials and standards

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (ACN), methanol (MeOH), ethyl acetate (EtAc) and formic acid (FA) were obtained from Fischer Scientific (Loughborough, UK). Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA) and was used for preparing the mobile phase and other aqueous solutions. Anhydrous magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), sodium citrate tribasic dehydrate, sodium citrate dibasic sesquihydrate and 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent (FR, 2N) was from Fluka (Buchs, Switzerland) and anhydrous sodium carbonate was obtained from Panreac (Barcelona, Spain). Sorbents (50 µm particle size) for *d*SPE, including primary-secondary amine (PSA), anhydrous $MgSO_4$ and trifunctionally-bonded C18 silica, were obtained from Waters (Milford, MA, USA).

Gallic acid monohydrate (98%), ferulic acid (98%), epicatechin (≥ 95%), *p*-coumaric acid (99%), rutin (≥ 95%), kaempferol (≥ 97%), protocatechuic acid (98%), chlorogenic acid (≥ 95%), naringenin (≥95%) and *trans*-resveratrol (99%) were purchased from Sigma-Aldrich (St.

Louis, MO, USA), whereas myricetin (≥ 97%) and 4-hydroxybenzoic acid (≥ 99%) were from Acros Organics (Geel, Belgium).

2.2. Preparation of standard solutions

Individual stock standard solutions (1000 μg mL^{-1}) were prepared in MeOH and stored at -20 ºC in darkness. Multicomponent standard solutions of 20 μg mL^{-1} were prepared by dilution of each primary standard solution in different solvents (ACN, MeOH, EtAC, ACN:MeOH (1:1, v/v) and ACN:EtAc (1:1, v/v), all of them containing 0.1% FA) and were used to optimize the extraction conditions. For validation purposes, working standard solutions containing the target analytes at different concentration levels were prepared daily by dilution of the individual stock solutions with ACN:EtAc (1:1, v/v) containing 0.1% FA. The target polyphenols were chosen based on their importance and relevance on food quality, involving the major classes (flavonoids and non-flavonoids).

2.3. Baby food samples

Nine different commercial baby foods including porridges, juices and pureed solids, were purchased from local pharmacies and supermarkets in Funchal, Portugal. Their declared ingredients according to their labels are given in Table 1. The samples were grouped based on their composition, and were divided into two groups: samples with high content of sugars (fruit-based baby foods) and samples with high content of fats and proteins (meat and vegetables baby foods). In this sense, for validation purposes, the sample of "multi-fruits with cereals" was chosen as representative sample of the baby foods with high content in sugars, since many of its ingredients were present in the other fruit-based samples, and the "chicken, beef and vegetables" baby food was chosen as representative sample of the baby foods with high content in fats and proteins, since it has similar ingredients to the other baby food samples with meat and vegetables in their composition. The samples were kept at 4 ºC until they were subjected to the μ-QuEChERS extraction procedure without any previous pre-treatment step, and all of

them were extracted and analyzed in triplicate. To determine the TPC and the antioxidant activity of the baby foods, the samples were subjected to a maceration process in order to obtain a liquid extract. For this purpose, 25 g of sample were mixed with 50 mL of MeOH:H_2O (95:5, v/v) acidified with 0.1% FA, the mixture was kept in maceration for 24 hours in darkness, it is important to consider that under these conditions most of the glycosides forms could be converted in their corresponding aglycones. After maceration, the sample extracts obtained were filtered under vacuum and stored at 4 ºC until TPC and RSA analysis.

2.4. μ-QuEChERS

The μ-QuEChERS procedure was adapted from the methodology reported by Porto-Figueira et al. [14], which is a miniaturization of the original QuEChERS procedure proposed by Anastassiades et al. [8]. First, the salts for μ-QuEChERS mixture (anhydrous $MgSO_4$, NaCl, sodium citrate tribasic dehydrate and sodium citrate dibasic sesquihydrate) were weighted keeping the original proportion 4:1:1:0.5. All the salts were mixed and homogenized in a vortex to achieve a visually homogeneous mixture. In order to get the highest extraction efficiency, different partitioning solvents (MeOH, ACN and EtAc) combined in different proportions were tested and compared. For clean-up procedure by *d*SPE, the use of $MgSO_4$ and PSA with and without C18 was investigated, and different reconstitution volumes (100 and 150 μL) were also evaluated.

The μ-QuEChERS procedure performed in this work was as follows: 0.3 g of sample were directly weighted into a 2 mL centrifuge tube with screw cap, then 0.2 g of the μ-QuEChERS mixture (buffered salts) and 1 mL of ACN:EtAc (1:1, v/v) containing 0.1% FA were added. The tube was vortexed for 10 s, followed by ultrasound agitation for 5 min and centrifuged 5 min at 5000 rpm. An aliquot (700 μL) from the upper part of the extract was transferred into a 2 mL PTFE *d*SPE clean-up tube containing: 75 mg of $MgSO_4$ and 12.5 mg of PSA. The mixture was vortexed for 30 s and centrifuged 5 min at 4000 rpm. Then, 500 μL of the purified extract

were filtered through a 0.22 μm PTFE filter membrane and evaporated under a nitrogen stream to dryness. Finally, the residue was reconstituted in 100 μL of MeOH for subsequent analysis on the UHPLC-PDA system.

2.5. UHPLC-PDA analysis and operating conditions

The chromatographic analysis of polyphenols was performed on a Waters Ultra-High Pressure Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) (Milford, MA, USA) equipped with a Water Acquity quaternary solvent manager (QSM), a column heater, an Acquity sample manager (SM), a 2996 PDA detector, and a degassing system. Separation was achieved with an Acquity HSS T3 analytical column packed with a trifunctional C18 alkyl phase (2.1 mm × 100 mm, 1.8 μm particle size). The column oven temperature was set at 40 ºC. A binary mobile phase with a gradient program was used, combining solvent A (water containing 0.1% FA) and solvent B (MeOH) as follows: 80% A (0 min), 80-60% A (3 min), 60-55% A (3 min), 55-30% A (2 min), 30-55% A (2 min), 55-80% A (2 min). The system was re-equilibrated with the initial composition for 2 min prior to next injection; yielding a total analysis time of 14 min. The flow rate was 250 μL min^{-1}, the injection volume was 2 μL and samples were kept at 20 ºC during the analysis. The UV detection wavelength was set to the maximum of absorbance for the target analytes (Table 2), and the identification of polyphenols was based on the comparison of the retention times (Table 2) and PDA spectra of their peaks in the samples with those previously obtained by the injection of pure standards.

2.6. Analytical method validation

The proposed μ-QuEChERS procedure was properly validated in terms of selectivity, linear dynamic range (LDR), detection and quantification limits, intra-day and inter-day precision and accuracy. Also, matrix effect (ME) of the extraction procedure were evaluated. For method validation the “multi-fruits with cereals” and the “chicken, beef and vegetables” baby foods

were used as matrices to calculate the validation parameters of both groups of samples (samples with high content of sugars and samples with high content of fat and proteins, respectively).

Selectivity was assessed by the absence of interfering or co-eluting chromatographic peaks at the retention time of the target analytes in the samples extracted by the optimized μ-QuEChERS method, and by checking the PDA spectra and purity of the peaks obtained. Matrix-matched calibration curves were constructed to evaluate the LDR of the method. For this purpose, the representative baby foods of each group of samples were spiked at six concentration levels and were extracted by the optimized μ-QuEChERS method. The concentration ranges were selected according to the sensitivity of the UHPLC-PDA system towards each target analyte. Calibration curves were obtained by plotting the average peak area of each analyte against the analyte concentration, and were fitted by linear least-square regression. Solvent-based standard calibration curves were used to assess ME by using working standard solutions prepared and analyzed using the optimized μ-QuEChERS method. The ME was studied through the comparison between the slopes of the matrix-matched and solvent-based standard calibration curves. The quotient between the slopes shows the ME value, a value of 100 ± 20% indicates the matrix does not significantly influence the extraction efficiency, on the other hand, a value higher than 120% indicates matrix enhancement, while a value lower than 80% indicates the matrix negatively hinders the extraction of the target analytes. The method detection limits (MDLs), defined as the lowest analyte concentration that produces a response detectable above the noise level of the system, and method quantification limit (MQLs), known as the lowest level of analyte that can be accurately and precisely measured, were calculated for each compound considering the concentration that produced a signal-to noise ratio (S/N) equal or higher than 3 and 10, respectively, using the lowest concentration of the matrix-matched calibration curve. The accuracy was expressed as recovery percentage (%) and was assessed by spiking the representative baby foods of each group of samples in triplicate at three concentration levels (low, medium and high) and subjecting them to the μ-QuEChERS

procedure. The recovery values were determined by comparison of the areas of the spiked samples with the areas of simulated samples (samples spiked at the same concentration levels but at the end of the extraction process prior to UHPLC-PDA analysis). Precision (expressed as relative standard deviation, RSD %) was evaluated in terms of intra-day (repeatability) and inter-day (reproducibility) precision using the same fortification levels than for the accuracy assays. Six replicates (n = 6) of the whole procedure for each sample were performed on the same day, by the same analyst to obtain intra-day precision. For inter-day precision, six replicates of each level were analyzed daily through three different days (n = 18).

2.7. Total polyphenolic content and antioxidant activity determination

The TPC of the sample extracts was determined by the Folin-Ciocalteu's colorimetric method described by Lim et al. [15]. Briefly, 0.6 mL of each extract, 3 mL of Folin-Ciocalteu reagent (previously diluted 10 times with deionized water) and 2.4 mL of 7.5 % (w/v) of sodium carbonate were added and mixed thoroughly with a vortex. The reaction mixture was kept in darkness during 30 min and then its absorbance was measured at 765 nm. The estimation of TPC in the extracts was calculated by a calibration curve obtained with gallic acid (from 20 to 150 mg L^{-1}), and were expressed as mg of gallic acid equivalents (GAE) per kg of sample. All measurements were performed in triplicate.

For antioxidant activity, the DPPH$^{\bullet}$ free RSA of the sample extracts was determined as previously reported by Brand-Williams et al. [16] and Perestrelo et al. [17] with some modifications. Briefly, 100 μL of the sample extracts were mixed with 3.9 mL of methanolic solution of DPPH$^{\bullet}$ (0.075 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min, and then its absorbance was measured at 515 nm. The RSA was calculated as percentage of DPPH$^{\bullet}$ discoloration of the extracts using the following formula and expressed as inhibition %:

$$RSA\ (\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the DPPH$^{\bullet}$ solution and A_{sample} is the absorbance of the sample extract. A calibration curve was obtained using a gallic acid standard solution at different concentrations (from 20 to 150 mg L^{-1}). The results obtained were also expressed as mg GAE per kg of sample. All determinations were carried out in triplicate.

3. Results and discussion

3.1. Optimization of μ-QuEChERS

The μ-QuEChERS procedure was based on the original method proposed by Anastassiades et al. [8] with addition of citrate buffer to preserve pH during extraction, and it was miniaturized by a significant reduction of partitioning salts, organic solvents and sample amount.

Firstly, some preliminary studies were carried out with standard solutions using 0.20 g of partitioning salts in the proportion 4:1:1:0.5 (anhydrous $MgSO_4$, NaCl, sodium citrate tribasic dehydrate and sodium citrate dibasic sesquihydrate), ACN as extraction solvent, 0.10 g of clean-up sorbents (75 mg $MgSO_4$, 12.5 mg PSA and 12.5 mg C18) and MeOH as reconstitution solvent, since it was the organic solvent used for in the mobile phase gradient. Under these conditions different reconstitution volumes (100 and 150 μL) were tested in order to evaluate the behavior of the target analytes. Results showed that 100 μL of MeOH were enough to recover the analytes providing good signal on the UHPLC-PDA, therefore this volume was selected for reconstitution purposes. Afterwards, different extraction solvents and mixtures (ACN, MeOH, ACN:MeOH (1:1, v/v), EtAc and ACN:EtAc (1:1, v/v), all of them containing 0.1% FA) and different types of sorbents (PSA and C18) for the clean-up step were assayed and evaluated using 0.20 g of partitioning salts in the proportion 4:1:1:0.5 and 100 μL of MeOH as reconstitution volume. PSA helps to remove compounds such as polar organic acids, polar pigments, sugars and fatty acids, therefore its use is indicated for the clean-up of samples with this type of substances in their composition, such as fruits and vegetables, while C18 removes non-polar interfering substances like lipids, thus the combination of PSA and C18 sorbents is

indicated for a more efficient clean-up procedure in fatty and complex matrices [18]. Since the baby food samples were classified into samples with high content of sugars and samples with high content of fats and proteins, the use of $MgSO_4$ and PSA with and without C18 in the clean-up procedure was investigated. Fig. 1 shows the total peak areas obtained under the different extraction conditions evaluated. In many cases, ACN is selected as extraction solvent because it affords protein precipitation, what can be considered a first clean-up step since it provides extracts with less interfering substances than other organic solvents [19, 20]. However, according to the results obtained, ACN was the solvent with less extraction capability. As it can be observed (Fig. 1), when using the clean-up mixture without C18 (75 mg $MgSO_4$ and 12.5 mg PSA) the combination of ACN:EtAc (1:1, v/v) was clearly the most efficient solvent, therefore these extraction conditions were selected to extract the target polyphenols from the baby food matrices with high content of sugar. On the other hand, when using C18 in the clean-up procedure (75 mg $MgSO_4$, 12.5 mg PSA and 12.5 mg C18), which is recommended for samples with high content of fats and proteins, the solvents containing MeOH and EtAC 100% showed similar extraction values among them, with no big differences (Fig. 1). However, it is well-known that MeOH is not a good clean-up solvent, since it extracts many interfering matrix components. Therefore, in order to determine if the combination of PSA and C18 in the clean-up step really improves method performance and helps to obtain cleaner sample extracts from fatty and complex matrices, a recovery study was carried out using this baby food sample spiked with a known amount of the target polyphenols, applying both µ-QuEChERS procedures: µ-QuEChERS procedure 1 (without C18 and ACN:EtAc (1:1, v/v) as extraction solvent) and µ-QuEChERS procedure 2 (with C18 and EtAc 100% as extraction solvent). The recovery values were determined by comparison with simulated samples. Results in Fig. 2 show, in general, a similar extraction capability of both procedures, except in the case of gallic acid, chlorogenic acid, rutin and myricetin. Although the use of C18 sorbent is indicate for the clean-up of complex matrices, according to the results, it was not suitable for the extraction of these

compounds, since it provided lower recovery values than the ones obtained without using C18 in the clean-up mixture, probably because these compounds remain retained in the C18 sorbent. Therefore, the best experimental conditions selected for the determination of the target polyphenols in baby food matrices with high content of fats and proteins using the developed μ-QuEChERS method were ACN:EtAc (1:1, v/v) as extraction solvent and clean-up without C18 (75 mg $MgSO_4$ and 12.5 mg PSA), which are the same conditions than the ones selected for the baby food matrices with high content of sugars.

3.2. Method validation

To demonstrate the feasibility and practicability of the proposed μ-QuEChERS approach for quantification of polyphenols in baby food samples, the performance of the method was validated in terms of selectivity, linearity, MDL, MQL, intra/inter-day precision and accuracy, in both types of samples: with high content of sugars (fruit-based baby foods) and with high content of fats and proteins (baby foods with meat in their composition), using the representative samples selected for each group. The validation parameters are shown in Tables 3 and 4.

Regarding method selectivity, no interfering peaks (different from the ones of the target analytes) were detected at the retention time of the target analytes at their maximum absorption quantification wavelengths, this was also checked by the PDA spectra and the purity of the peaks observed. For linearity, matrix-matched calibration curves were obtained by plotting peak areas versus analyte concentrations in the spiked baby food samples prepared and analyzed under the optimized extraction conditions. Along with each calibration curve a zero sample (sample without spiking) was also analyzed. The least-squares linear regression analysis of the data provided excellent linearity with correlation coefficients (R^2) values >0.99 in the concentration range studied for both samples (Tables 3 and 4). Moreover, to evaluate the existence of ME that may affect the extraction of analytes, the LDR of the method was also

established on solvent-based standard calibration curves (R^2 > 0.99 for all compounds, Table 2). ME expressed as the matrix/solvent slope ratios were calculated for each analyte in both types of matrices. According to what has been previously explained in Section 2.6, results showed that, in general, ME were higher in the sample with high content of sugars (4 analytes without matrix interferences) (Table 3) than in the sample with high content of fats and proteins (8 analytes without interferences) (Table 4). In both matrices, chlorogenic acid showed a reduction in the slope due to ME, while kaempferol showed a sharp increase of the slope caused by ME. On the other hand, gallic acid did not show ME in the sample with high content in sugar, while in the sample with high content of fats and proteins its extraction was strongly negatively affected by the matrix. Significant differences were also observed in the case of myricetin, which extraction was really enhanced by the matrix with high content of sugar, while in the other matrix no ME were observed in its extraction. Therefore, in order to compensate the errors associated ME, matrix-matched calibration curves should be used for quantification purposes of the target analytes in the baby food samples. MDLs and MQLs were calculated based on threefold and tenfold signal to noise (S/N) ratios, respectively, obtained from the lowest concentration level of the calibration curves. MDLs and MQLs ranged between 0.04 – 0.46 and 0.13 – 1.54 μg g^{-1} for samples with high content of sugars (Table 3) and between 0.03 - 0.56 and 0.10 – 1.85 μg g^{-1} for samples with high content of fats and proteins (Table 4), respectively, hence the method is enough sensitive for the determination of low levels of polyphenols in baby food samples. Nonetheless, gallic acid showed significant higher MDLs and MQLs than the other analytes, this could be due because this compound elutes very close to the dead volume of the system, what may negatively affect the quantification reliability of this analyte.

The accuracy and precision of the method were evaluated for both matrices at three concentration levels (low, medium and high) and results are summarized in Tables 3 and 4. Regarding accuracy of the proposed method, results at the three different concentration levels

were almost in the same range and satisfactory average recovery values were achieved in both matrices, ranging from 73 – 100 % in samples with high content of sugars (Table 3) and 71 – 100 % in samples with high content of fats and proteins (Table 4). Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility, and results are represented by the RSD % at each fortification level for each compound. Satisfactory results were achieved with RSD values lower than 5 % for intra-day precision and lower than 6 % for inter-day precision in both matrices (Tables 3 and 4), indicating the strong stability of the developed method.

3.3. Application of μ-QuEChERS/UHPLC-PDA methodology for the analysis of polyphenols on baby food samples

The developed method was applied for the determination of the target polyphenols in nine different commercial baby foods (including porridges, juices and pureed solids). Each sample was analyzed in triplicate. The areas of the compounds that were clearly recognized by their PDA spectrum and retention time were interpolated in the corresponding matrix-matched calibration curve for quantification purposes, according to their classification based on their composition (Table 1). Table 5 summarizes the results obtained for all the samples analyzed. As it can be observed, the profile and concentrations of the analytes varied among the different matrices, and not all were detected in each sample. Rutin, *trans*-resveratrol and myricetin were not detected in any of the samples analyzed, and other polyphenols were detected at concentrations levels lower their MQL so they could not be quantified, such as epicatechin (except in the vegetables and beef sample) and *p*-coumaric acid. Generally, gallic acid, 4-hydroxybenzoic acid and naringenin were the main polyphenols detected in all the samples analyzed. Naringenin is a flavanone with high chemopreventive and therapeutic potential which is usually abundant in citrus fruits such as orange and lemon [21 – 23]. This polyphenol was detected and quantified in all the baby foods analyzed, as it can be observed in the chromatograms of all the samples measured at 289 nm, which the maximum quantification

wavelength for naringnenin (Fig. 3). Therefore, the occurrence of this compound in many of the samples could be due to the addition of concentrated lemon and orange juice in their composition (Table 1). The fruit-based baby food samples presented higher amount of polyphenols than the baby foods with meat and vegetables in their composition, except the porridges, which despite containing fruits as ingredients of their formulation, they showed similar values to the latter ones. In the fruit-based baby foods, the main polyphenols quantified were gallic acid, protocatechuic acid and chlorogenic acid, while the presence of other compounds, such as epicatechin, *p*-coumaric acid and ferulic acid was detected at levels lower than their MQL. These results agree with the fact that these compounds are the main polyphenols that can be found in fruits, particularly in bananas and apples [23 – 25]. Chlorogenic acid was the main polyphenol quantified in the samples containing apple (apple juice, apple pureed and multi-fruits with cereals pureed), since according to previous reports is the main polyphenol found in this fruit [23, 24]. Moreover, a significant amount of gallic acid was also quantified in the "apple juice", which is also one of the predominant polyphenols found in apples [24]. The levels of polyphenols found in the porridge samples were lower than the ones found in the fruit-based solid pureed baby foods, probably because these samples have less percentage of fruits in their composition (Table 1), and because they are more processed products, therefore the technological processes to which they are subjected may have a detrimental impact on these compounds. In this sense, in the "gluten-free dairy-fruits porridge" only gallic acid and 4-hydroxybenzoic acid were quantified, which are basic units of polyphenols. Naringenin was also quantified, probably because of the addition of concentrated lemon and orange juice in its composition. On the other hand, in the "8 cereals with honey porridge", apart from naringenin, only 4-hydroxibenzoic acid was quantified, which is one of the main polyphenols found in honey [4], and ferulic acid was detected at low levels, probably due to the presence of cereals as ingredients of the sample, since it is the most abundant polyphenol in cereals [22, 23].

In the pureed solid baby foods with meat in their composition, the levels of polyphenols were also lower than the ones obtained in the fruit-based baby foods, probably because these samples are also subjected to more technological processes such as cooking treatment that may affect and reduce the amount of these compounds in comparison to their concentration in raw materials. Apart from naringenin, 4-hydroxybenzoic acid, ferulic acid and kaempferol were the polyphenols quantified in all samples of this type. Kaempferol is one of the main polyphenols found in onions, while the presence of ferulic acid is probably due to cereals [23]. Gallic acid and chlorogenic acid were detected in these samples at levels lower than their MQL, so it was not possible to accurately quantify them, however, the occurrence of chlorogenic acid was probably due to the presence of carrots and potatoes as ingredients, since is one of the main polyphenols found in these vegetables [22, 23, 26, 27].

3.4. Antioxidant potential of baby food samples

The TPC and RSA of the studied baby food samples are indicated in Table 5. The results were significantly different among the different samples; the TPC varied from 1030 to 8054 mg GAE per kg of sample and the antioxidant capacity ranged from 155 to 5767 mg GAE per kg of sample, which are values higher than the ones reported by other authors for fruit baby foods [3]. The highest values of TPC were obtained in the porridges; however, their RSA was lower, and according to the UHPLC-PDA results these samples had fewer amounts of polyphenols than the other fruit-based baby food samples assayed. Therefore, since there was no correlation between the TPC and RSA values of these samples, the high values and the differences observed in the TPC of the porridges could be explained by the presence of sugars that may interfere in the measurement of polyphenols at the assayed wavelength, since porridges are products with high content of sugar in their composition (Table 1). On the other hand, the TPC and antioxidant capacity RSA were fairly well correlated in the solid fruit-based pureed and the apple juice samples (Table 5), and according to the UHPLC-PDA results these fruit-based samples were

the ones with the highest amount of polyphenols, so it was determined that polyphenols were the main compounds responsible for the RSA of these samples. As in the previous UHPLC-PDA results, the lowest values of TPC and RSA were found in the solid pureed baby foods samples with meat in their composition (Table 5). Although these samples have a high percentage of vegetables in their composition (64 – 85 %) (Table 1) which can contribute to the occurrence of polyphenols with antioxidant activity, they are products that have been subjected to a cooking process that may produce a negative impact on these compounds reducing their amount, and consequently their RSA too. Overall, according to the obtained results, it was concluded that the highest RSA as well as the content of polyphenols were found in the fruit-based baby food samples.

4. Conclusions

In this work, an improved, quick, simple, sensitive and reliable analytical method based on the miniaturization of the QuEChERS extraction technique combined with UHPLC-PDA was developed for the simultaneous determination of twelve polyphenols in different baby food samples. The proposed analytical approach proved to be an improved environmentally friendly strategy, since it showed good extraction efficiency using fewer amounts of sample, organic solvents, clean-up sorbents and partitioning salts than the original method. This methodology also reduces the time and cost of the analysis, since it requires little sample preparation and provides adequate clean-up that simplifies the detection and quantification of polyphenols in different baby food matrices. The method was successfully validated for two different types of baby food matrices (with high content of sugars and with high content of fats and proteins). The applicability of the method was shown by the analysis of nine commercial baby food samples, including porridges, solid purees and juices, what allowed characterizing the abundance of the selected polyphenols in these products. Moreover, the TPC and RSA of all the samples were investigated, and it was concluded that the highest RSA as well as the content of polyphenols

were found in the fruit-based baby foods. Thus, this work represents a first approach to determine and evaluate the nutritional quality of this kind of products.

Acknowledgments

Authors thank the Rey Juan Carlos University for providing Natalia Casado a mobility grant to perform a pre-doctoral stay in the CQM-Chemistry Center of Madeira. Rosa Perestrelo and Catarina Silva acknowledge the FCT-Fundação para a Ciência e a Tecnologia for their pos-Doc and PhD grants, SFRH/BPD/97387/2013 and SFRH/BD/97039/2013, respectively. The authors also acknowledge the support of FCT under CQM grant PEst-OE/QUI/UI0674/201. Funding through the project M1420-01-0145-FEDER-000005 - Centro de Química da Madeira CQM^{+} (Madeira 14-20) is also acknowledged.

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Figure Captions

Fig. 1 Evaluation of different extraction solvents on the extraction efficiency of the target polyphenols from standard solutions by the µ-QuEChERS procedure using different clean-up sorbent mixtures: $MgSO_4$ and PSA with or without C18. Error bars represent the standard deviation of samples replicates (n=3).

Fig. 2 Recovery percentages of the target polyphenols obtained from a spiked baby food sample with high content of fats and proteins extracted under different conditions: µ-QuEChERS procedure 1 ($MgSO_4$ + PSA and ACN:EtAc (1:1, v/v) as extraction solvent) and µ-QuEChERS procedure 2 ($MgSO_4$ + PSA + C18 and EtAc 100% as extraction solvent). Error bars represent the standard deviation of samples replicates (n=3).

Fig. 3 Extracted chromatograms of the target analytes at 289 nm (quantification wavelength of naringenin) in all the baby foods analysed using the chromatographic conditions described in section 2.5. The chromatograms numbers correspond to the following samples: 1- Gluten free dairy porridge with fruits; 2- 8 Cereals and honey porridge; 3- Apple pureed; 4- Banana pureed; 5- Chicken, beef and vegetables pureed; 6- Vegetables and beef pureed; 7- Lamb stew pureed; 8- Multi-fruits with cereals pureed; 9- Apple juice.

Fig. 1

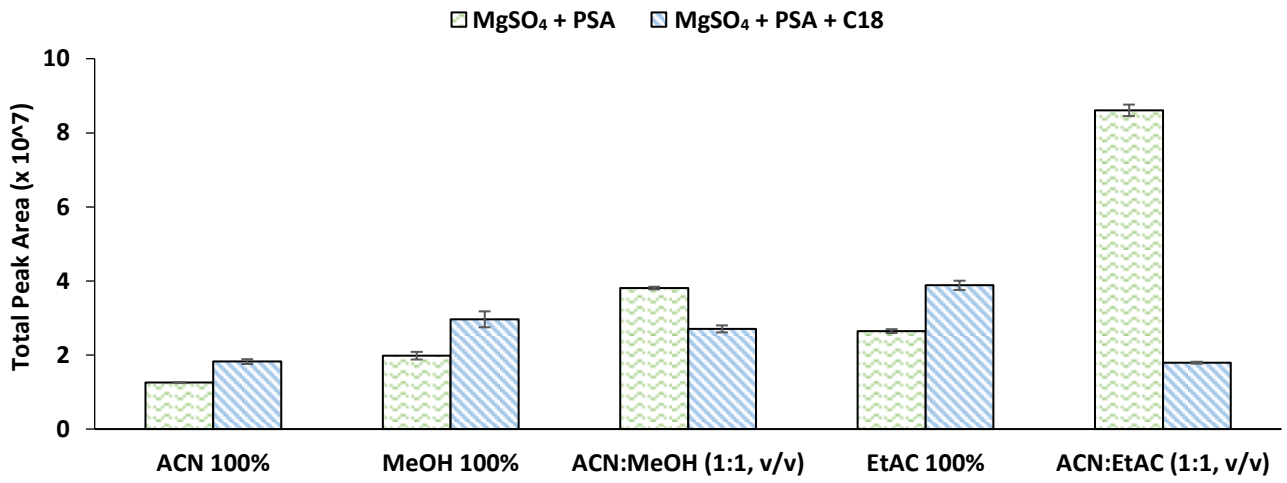
MgSO4 + PSA
MgSO4 + PSA + C18
Total Peak Area (x 10^7)
10
8
6
4
2
0
ACN 100%
MeOH 100%
ACN:MeOH (1:1, v/v)
EtAC 100%
ACN:EtAC (1:1, v/v)

Fig. 2

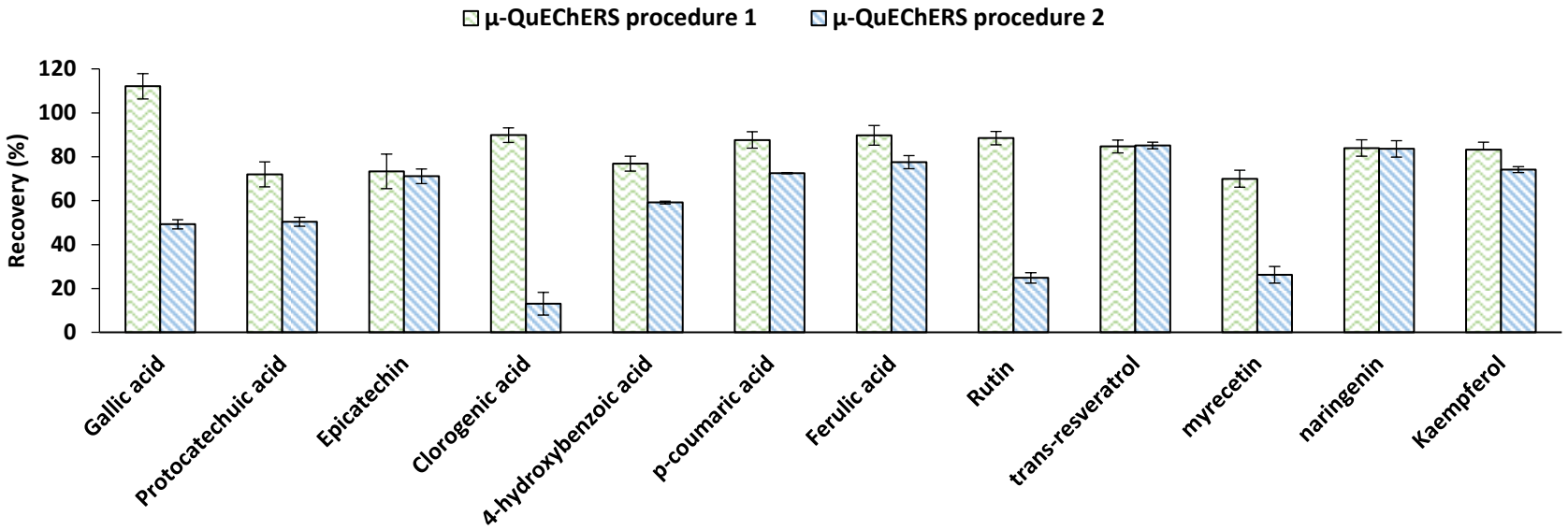
μ-QuEChERS procedure 1
μ-QuEChERS procedure 2
Recovery (%)
120
100
80
60
40
20
0
Gallic acid
Protocatechuic acid
Epicatechin
Clorogenic acid
4-hydroxybenzoic acid
p-coumaric acid
Ferulic acid
Rutin
trans-resveratrol
myrecetin
naringenin
Kaempferol

Fig. 3

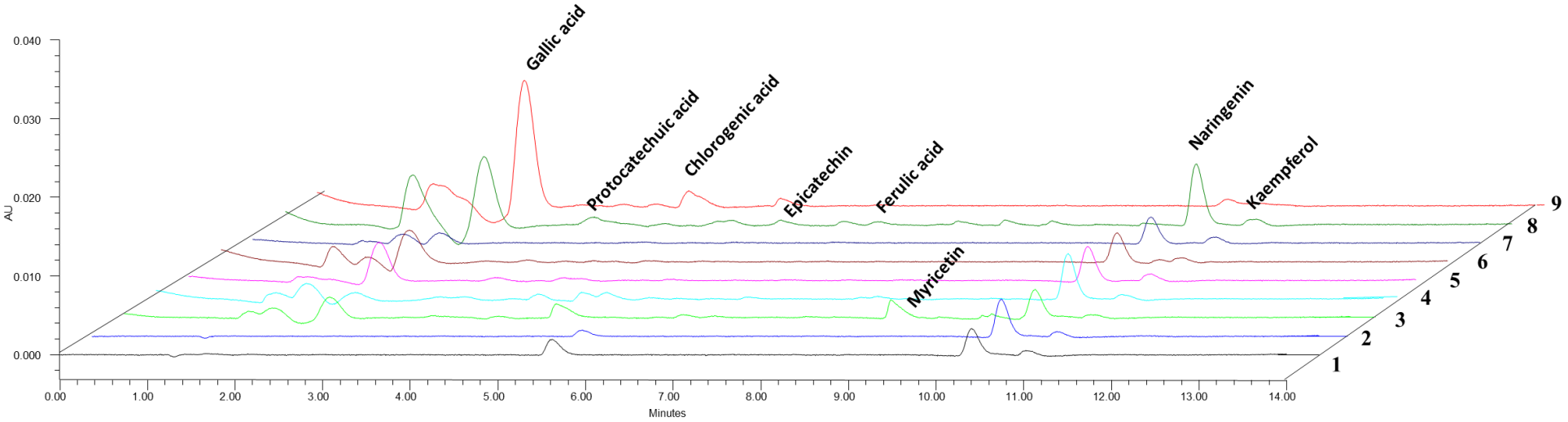

Table 1. Declared ingredients for the commercial baby food samples analyzed.

Sample classification	Baby food samples	Ingredients
High content of sugars	Banana pureed	Banana (100%), concentrated lemon juice and ascorbic acid.
	Apple pureed	Apple (94%), corn starch, rice and ascorbic acid.
	Multi-fruits with cereals pureed	Fruits 96% (apple, banana, peach, orange juice, apricot and pear), cereals 4% (corn and rice) and ascorbic acid.
	Apple juice	Concentrated apple juice and vitamin C
	Gluten free dairy porridge with fruits	Cereals 29% (corn starch, rice flour), fruits 29% (apricot, peach, banana, concentrated orange juice and concentrated lemon juice), milk proteins, sugar, skim milk powder 10%, palm oil, minerals (calcium, phosphorus, iron), vitamins (C, niacin, E, pantothenic acid, B_1, B_2, B_6, A, folic acid, K_1, biotin, D_3 and B_{12}), soy lecithin and vanilla.
	8 cereals and honey porridge	Cereals 51% (wheat flour, barley flour, rye flour, rice flour, corn starch, oatmeal, indian cornmeal, sorghum flour), sugar, maltodextrin, honey 5%, fructo-oligosaccharides 2%, palm oil, minerals (calcium and phosphorus), soy lecithin, vitamins (C, niacin, E, pantothenic acid, B_1, B_6, A, folic acid, K_1, biotin and D_3) and vanilla.
High content of fats and proteins	Chicken, beef and vegetables pureed	Vegetables 85% (potatoes, tomatoes, carrots, and onions), cooking water, meat 15% (chicken and beef), rice, corn starch, lemon juice, salt and olive oil (0.6%).
	Lamb stew pureed	Vegetables 63.9% (potatoes, carrots, green beans, onions, and green peas), cooking water, lamb (8%), corn starch and extra virgin olive oil (1.3%).
	Vegetables and beef pureed	Vegetables 85% (green beans, carrots, onions, potatoes), beef (8%), cooking water, rapeseed oil, sunflower oil and salt.

Table 2 Retention time (RT), maximum wavelength, linear dynamic range (LDR) and linearity of the twelve target polyphenols calculated using the proposed µ-QuEChERS/UHPLC-PDA methodology with standard solutions.

Phenolic compounds	**RT[a] (min)**	**Wavelength (nm)**	**LDR[b] (µg mL^{-1})**	**Linearity, R^2**
Gallic acid	2.1	271	1.00 – 8.00	$y = 1.0\times10^5\,x - 5.0\times10^5$ 0.998
Protocatechuic acid	3.1	259	0.40 – 6.00	$y = 7.7\times10^4\,x - 5.4\times10^4$ 0.998
Chlorogenic acid	4.1	326	0.10 – 3.00	$y = 9.5\times10^4\,x - 2.6\times10^5$ 0.999
4-Hydroxybenzoic acid	4.5	255	0.40 – 8.00	$y = 1.9\times10^5\,x - 4.5\times10^4$ 0.997
Epicatechin	5.0	278	0.20 – 6.00	$y = 2.2\times10^4\,x - 6.0\times10^3$ 0.992
p-Coumaric acid	6.1	309	0.10 – 3.00	$y = 1.9\times10^5\,x + 2.6\times10^3$ 0.999
Ferulic acid	6.5	323	0.10 – 3.00	$y = 1.3\times10^5\,x + 5.1\times10^3$ 0.999
Rutin	8.0	354	0.20 – 6.00	$y = 3.6\times10^4\,x - 5.6\times10^4$ 0.993
Trans-Resveratrol	8.3	305	0.10 – 3.00	$y = 2.3\times10^5\,x - 8.1\times10^3$ 0.997
Myricetin	9.1	372	0.10 – 3.00	$y = 6.7\times10^4\,x - 6.7\times10^4$ 0.997
Naringenin	10.1	289	0.20 – 6.00	$y = 1.3\times10^5\,x - 6.8\times10^2$ 0.995
Kaempferol	10.9	363	0.20 – 6.00	$y = 6.6\times10^4\,x + 3.5\times10^4$ 0.996

[a] RT – Retention time
[b] LDR – linear dynamic range

Table 3 Validation parameters of the µ-QuEChERS/UHPLC-PDA methodology for the determination of the target polyphenols in baby food samples with high content of sugars in their composition.

Phenolic compounds	Linear range (μg g^{-1})	Linearity, R^2 Matrix-matched calibration	Spiked levels (μg g^{-1})	Accuracy		Precision		MDL[a] (μg g^{-1})	MQL[b] (μg g^{-1})	ME[c] (%)
				Recovery (% ± sd)	Average Recovery (% ± sd)	Intra-day precision (RSD %)	Inter-day precision (RSD %)			
Gallic acid	1.50 - 30	$y = 9.7\times10^4\ x - 5.0\times10^4$ 0.993	1.50 15.0 30.0	69 ± 1 79 ± 1 70 ± 2	73 ± 5	2.8 2.1 2.6	4.0 3.9 3.4	0.46	1.54	96
Protocatechuic acid	0.10 - 20	$y = 1.0\times10^5\ x - 1.8\times10^4$ 0.993	0.10 10.0 20.0	73 ± 2 88 ± 2 82 ± 2	81 ± 7	4.1 2.8 2.5	4.5 3.2 3.1	0.05	0.17	130
Chlorogenic acid	0.30 - 20	$y = 2.7\times10^4\ x - 1.0\times10^5$ 0.999	0.30 10.0 20.0	77 ± 2 76 ± 1 74 ± 2	76 ± 1	3.0 1.8 2.3	4.3 3.0 3.7	0.10	0.33	28
4-Hydroxybenzoic acid	0.10 - 20	$y = 2.5\times10^5\ x + 1.9\times10^4$ 0.998	0.10 10.0 20.0	86 ± 1 83 ± 1 86 ± 2	85 ± 2	1.8 1.7 2.0	4.1 2.9 2.6	0.04	0.14	136
Epicatechin	0.70 - 20	$y = 1.4\times10^4\ x + 7.4\times10^4$ 0.991	0.70 10.0 20.0	85 ± 2 86 ± 1 87 ± 1	86 ± 1	3.9 2.9 3.1	4.8 3.5 3.6	0.23	0.77	64
p-Coumaric acid	0.10 - 10	$y = 1.9\times10^5\ x + 5.8\times10^4$ 0.996	0.10 5.0 10.0	89 ± 2 88 ± 2 78 ± 2	85 ± 6	1.6 2.9 2.1	2.2 3.2 3.4	0.05	0.15	98
Ferulic acid	0.20 - 20	$y = 1.6\times10^5\ x + 1.3\times10^4$ 0.995	0.20 10.0 20.0	84 ± 1 86 ± 3 87 ± 2	85 ± 1	2.0 3.3 2.1	5.1 4.8 4.2	0.07	0.23	125
Rutin	0.20 - 20	$y = 5.0\times10^4\ x + 8.9\times10^3$ 0.993	0.20 10.0 20.0	94 ± 2 104 ± 1 87 ± 2	95 ± 8	3.6 3.1 2.2	4.2 4.9 4.0	0.08	0.27	137
Trans-Resveratrol	0.10 - 10	$y = 2.3\times10^5\ x + 2.4\times10^3$ 0.998	0.10 5.0 10.0	82 ± 2 87 ± 3 85 ± 1	85 ± 2	3.5 3.1 1.5	5.5 5.2 3.1	0.04	0.13	100
Myricetin	0.10 - 10	$y = 1.1\times10^5\ x + 3.5\times10^3$ 0.993	0.10 5.0 10.0	86 ± 1 85 ± 1 87 ± 1	86 ± 1	4.2 2.4 1.3	5.3 5.0 2.4	0.06	0.19	165
Naringenin	0.30 - 20	$y = 1.3\times10^5\ x - 4.7\times10^4$ 0.998	0.30 10.0	94 ± 1 86 ± 1	91 ± 4	4.2 3.0	5.6 5.8	0.10	0.34	97

			20.0	93 ± 1		1.9	2.9				
Kaempferol	0.20 - 20	$y = 1.3\times10^5\ x - 2.0\times10^4$ 0.997	0.20 10.0 20.0	104 ± 3 98 ± 2 97 ± 2	100 ± 4	4.3 2.4 1.9	5.3 3.1 3.9	0.08	0.27	199

[a] MDL: method detection limit
[b] MQL: method quantification limit
[c] ME: matrix effect

Table 4 Validation parameters of the µ-QuEChERS/UHPLC-PDA methodology for the determination of the target polyphenols in baby food samples with high content of fats and proteins in their composition.

Phenolic compounds	Linear range (μg g^{-1})	Linearity, R^2 Matrix-matched calibration	Spiked levels (μg g^{-1})	Accuracy		Precision		MDL[a] (μg g^{-1})	MQL[b] (μg g^{-1})	ME[c] (%)
				Recovery (% ± sd)	Average Recovery (% ± sd)	Intra-day precision (RSD %)	Inter-day precision (RSD %)			
Gallic acid	1.80 - 30	$y = 1.5\times10^4\ x + 1.7\times10^5$ 0.990	1.80 15.0 30.0	101 ± 1 98 ± 2 100 ± 2	100 ± 1	4.0 2.8 2.8	5.1 4.2 4.0	0.56	1.85	15
Protocatechuic acid	0.10 - 20	$y = 7.9\times10^4\ x - 1.0\times10^4$ 0.994	0.10 10.0 20.0	105 ± 2 98 ± 2 98 ± 1	100 ± 4	2.6 3.0 1.6	4.2 5.2 2.7	0.04	0.12	103
Chlorogenic acid	0.30 - 20	$y = 6.5\times10^4\ x + 1.3\times10^4$ 0.992	0.30 10.0 20.0	97 ± 3 94 ± 2 99 ± 2	97 ± 2	4.1 2.7 2.7	5.1 4.2 4.1	0.11	0.35	69
4-Hydroxybenzoic acid	0.10 - 20	$y = 1.9\times10^5\ x - 4.5\times10^4$ 0.991	0.10 10.0 20.0	94 ± 1 91 ± 2 94 ± 2	93 ± 2	2.1 3.0 2.4	3.2 3.6 3.6	0.05	0.16	103
Epicatechin	0.40 - 20	$y = 2.0\times10^4\ x - 2.0\times10^3$ 0.992	0.40 10.0 20.0	87 ± 2 84 ± 2 84 ± 1	85 ± 2	4.1 2.2 1.6	5.2 4.2 3.0	0.13	0.43	91
p-Coumaric acid	0.10 - 10	$y = 2.1\times10^5\ x + 6.3\times10^3$ 0.995	0.10 5.0 10.0	103 ± 1 88 ± 3 83 ± 1	91 ± 10	1.8 3.8 1.7	3.2 4.1 3.2	0.03	0.10	112
Ferulic acid	0.10 - 10	$y = 1.7\times10^5\ x - 1.1\times10^4$ 0.998	0.10 5.0 10.0	99 ± 3 97 ± 2 96 ± 1	97 ± 1	3.5 2.8 2.6	4.1 3.8 3.5	0.03	0.11	132
Rutin	0.10 - 10	$y = 4.1\times10^4\ x - 3.3\times10^2$ 0.999	0.10 5.0 10.0	73 ± 2 86 ± 4 71 ± 1	77 ± 8	3.0 4.4 1.5	5.4 5.5 2.6	0.03	0.11	113
Trans-Resveratrol	0.10 - 10	$y = 2.0\times10^5\ x + 5.2\times10^3$ 0.999	0.10 5.0 10.0	91 ± 2 91 ± 3 93 ± 1	92 ± 1	2.0 3.3 2.0	4.8 4.2 3.6	0.05	0.18	86
Myricetin	0.10 - 10	$y = 6.6\times10^4\ x - 1.4\times10^3$ 0.999	0.10 5.0 10.0	73 ± 2 71 ± 1 70 ± 3	71 ± 1	2.7 3.2 3.4	3.9 5.2 4.9	0.04	0.14	98
Naringenin	0.30 - 20	$y = 1.1\times10^5\ x + 2.5\times10^3$ 0.992	0.30 10.0	90 ± 2 90 ± 1	89 ± 1	1.8 1.4	3.7 3.8	0.11	0.36	84

			20.0	88 ± 2		3.2	4.8			
Kaempferol	0.10 - 10	$y = 1.2\times10^5\ x - 1.1\times10^4$ 0.992	0.10 5.0 10.0	96 ± 1 97 ± 3 98 ± 2	97 ± 1	3.3 3.2 2.4	4.4 4.5 3.8	0.06	0.19	180

[a] MDL: method detection limit
[b] MQL: method quantification limit
[c] ME: matrix effect

Table 5 Abundance of the target polyphenols in the baby food samples analysed by the μ-QuEChERS/UHPLC-PDA methodology and TPC and RSA of the baby food extracts. Values expressed as mean ± sd (n=3).

Phenolic compounds	**Multi-fruits with cereals (μg g^{-1})**	**Banana (μg g^{-1})**	**Apple (μg g^{-1})**	**Apple juice (μg g^{-1})**	**Gluten free dairy-fruits (μg g^{-1})**	**8 cereals with honey (μg g^{-1})**	**Chicken, beef and vegetables (μg g^{-1})**	**Lamb stew (μg g^{-1})**	**Vegetables and beef (μg g^{-1})**
Gallic acid	8.0 ± 0.2	3.5 ± 0.1	4.2 ± 0.2	26 ± 5	2.45 ± 0.02	n.d.[a]	<1.85	<1.85	<1.85
Protocatechuic acid	1.8 ± 0.1	2.9 ± 0.1	1.034 ± 0.008	n.d.	n.d.	n.d.	n.d.	1.68 ± 0.05	n.d.
Chlorogenic acid	16.97 ± 0.06	n.d.	16.99 ± 0.04	32 ± 3	n.d.	n.d.	<0.35	<0.35	<0.35
4-Hydroxybenzoic acid	<0.14	<0.14	0.172 ± 0.004	n.d.	1.12 ± 0.01	0.370 ± 0.005	1.46 ± 0.02	1.186 ± 0.005	0.970 ± 0.006
Epicatechin	<0.77	<0.77	<0.77	n.d.	n.d.	n.d.	n.d.	n.d.	1.64 ± 0.09
p-Coumaric acid	<0.15	<0.15	<0.15	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.
Ferulic acid	<0.23	<0.23	<0.23	n.d.	n.d.	<0.23	0.26 ± 0.03	0.25 ± 0.01	0.255 ± 0.004
Naringenin	3.3 ± 0.7	3.1 ± 0.3	2.44 ± 0.02	1.8 ± 0.1	2.4 ± 0.2	3.02 ± 0.02	1.48 ± 0.03	1.28 ± 0.03	1.24 ± 0.07
Kaempferol	n.d.	n.d.	0.60 ± 0.08	n.d.	n.d.	n.d.	0.33 ± 0.01	0.348 ± 0.006	0.325 ± 0.002
TPC[b] (mg GAE kg^{-1} sample)	5191 ± 32	4581 ± 53	4172 ± 49	5540 ± 21	8045 ± 58*	7948 ± 71*	1030 ± 10	1819 ± 51	2563 ± 55
RSA[c] (mg GAE kg^{-1} sample)	4417 ± 41	4499 ± 90	3163 ± 16	5767 ± 23	2696 ± 41	1673 ± 96	219 ± 30	155 ± 26	323 ± 40

[a] n.d.- analyte non detected in the samples.
[b] TPC – Total Polypohenolic Content; *values overestimated due to sugar interferences in the measurement.
[c] RSA – Radical Scavenging Activity