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Comparison of high-throughput microextraction techniques, MEPS and μ-SPEed, for the determination of polyphenols in baby food by ultrahigh pressure liquid chromatography

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ABSTRACT

In this study, two different high-throughput microextraction techniques, microextraction by packed sorbents (MEPS) and micro solid phase extraction (μ-SPEed®), were evaluated and compared, regarding the performance criteria, for the isolation of polyphenols from baby foods prior to their determination by ultrahigh pressure liquid chromatography (UHPLC). To achieve the best performance, influential parameters affecting extraction efficiency (including type of sorbent, number of extraction cycles, pH, elution solvent and elution volume) were systematically studied and optimized. To enable an effective comparison, selectivity, linear dynamic range, method detection (LODs) and quantification limits (LOQs), accuracy, precision and extraction yields, were determined and discussed for both techniques. Both methods provided the analytical selectivity required for the analysis of polyphenols in baby foods. However, μ-SPEed® sample treatment in combination with UHPLC-PDA has demonstrated to be more sensitive, selective and efficient than MEPS. Appropriate linearity in solvent and matrix-based calibrations, very low LODs and LOQs, ranging between 1.37 and 13.57 $\mu g\,kg^{-1}$ and 4.57 – 45.23 $\mu g\,kg^{-1}$, respectively, suitable recoveries (from 67 to 97%) and precision (RSD values < 5%) were achieved for the selected analytes by μ-SPEed®/UHPLC-PDA. Finally, the validated methodologies were applied to different commercial baby foods. Gallic acid, chlorogenic acid, epicatechin, ferulic acid, rutin, naringenin and myricetin are the most dominant polyphenols present in the studied baby food samples. The proposed methodology revealed a promising approach to evaluate the nutritional quality of this kind of products.

1. Introduction

Good nutrition is a critical factor during early life stages to promote correct development of babies, therefore the quality and composition of their diet is essential to ensure their current and future health (Cámara, Amaro, Barberá, & Clemente, 2005; Pandelova, Lopez, Michalke, & Schramm, 2012). Nowadays, food safety of baby products is one of the priorities in the food field. However, the composition and nutritive quality of these products normally go unnoticed. In this sense, research regarding the content of phenolic compounds and their antioxidant activities in baby food is very limited (Casado, Perestrelo, Silva, Sierra, & Câmara, 2018; Čížková, Ševčík, Rajchl, & Voldřich, 2009; Li, Friel, & Beta, 2010). The nutritive value of baby foods directly depends on the raw materials that are used and on their elaboration processes. Thus, the baby food products based on fruits and vegetables can be an excellent source of polyphenols and other antioxidants compounds. It has been evidenced that long term consumption of diets rich in polyphenols may prevent and offer protection against the development of future diseases, such as cancer, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Del Rio et al., 2013; Joseph, Edirisinghe, & Burton-Freeman, 2016; Liu, 2013; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). In addition, multiple beneficial effects related to their consumption have also been reported, including anti-carcinogenic, anti-atherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic effects (Nichenametla, Taruscio, Barney, & Exon, 2006; Wang et al., 2006).

The large number of health benefits associated to the consumption of polyphenols has promoted the interest in the development of analytical methods for their determination. Recently, novel analytical

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procedures based on microextraction techniques were proposed for quantitative determination of phenolic constituents in different food matrices (Casado, Morante-Zarcero, & Pérez-Quintanilla, 2018). These miniaturized extraction techniques have gradually gained attention due to their many advantages over conventional analytical methods, such as the minimal use of organic solvents or even solvent-free procedures, the low amount of sample required and the user-friendly systems. In this sense, microextraction by packed sorbents (MEPS) has successfully been evaluated for extraction of polyphenols from wine (Gonçalves & Câmara, 2011; Gonçalves, Mendes, Silva, & Câmara, 2012; Gonçalves, Silva, Castilho, & Câmara, 2013; Silva, Gonçalves, & Câmara, 2012) and beer (Gonçalves, Alves, Rodrigues, Figueira, & Câmara, 2013) samples. This technique was developed by Abdel-Rehim, Altun, and Blomberg (2004) as a miniaturization of the conventional SPE, being this way more sensitive, quick and cost-effective with minimal exposure to organic solvents. In MEPS, about 1–4 mg of sorbent are packed inside a syringe as a plug or between the barrel and the needle as a cartridge. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. A wide range of sorbent materials can be used including silica based (C2, C8, C18), strong cation exchanger (SCX) using sulfonic acid bonded silica, HILIC carbon, restricted access material (RAM), polystyrene-divinylbenzene copolymer (PS-DVB) and molecular imprinted polymers (MIPs) (Yang, Said, & Abdel-Rehim, 2017). Another microextraction technique is the μSPEed®, which uses small sorbent particles of < 3 μm, instead of the 50–60 μm particles normally used in SPE and/or MEPS. These smaller particles provide higher surface area, and thus a more efficient sorption of the target analytes. The sorbent is tightly packed in a disposable needle equipped with a pressure-driven valve to withdraw samples in a single direction. This configuration allows a constant and high pressure (up to 1600 psi) single direction flow through the sorbent, achieving more efficient extractions of the target analytes (Baranowska, Hejniak, & Magiera, 2016; Nalewajko-Sieliwoniuk, Malejko, Mozolewska, Wołyniec, & Nazaruk, 2015). Several sorbents for μ-SPEed® are available, such as unmodified silica C18 and functionalized polymeric polystyrene-divinylbenzene (PS/DVB) that allow expanding the application of μ-SPEed® by using several sorbent chemistries. In a recent work, a μ-SPEed® method for the extraction of phenolic compounds in teas samples was successfully optimized and validated involving minimal sample pre-treatment and solvent usage (Porto-Figueira, Figueira, Pereira, & Câmara, 2015), and it revealed a great potential for its application to other phenolics and matrices with minor changes in the experimental layout described.

Therefore, since the analysis of polyphenols in baby foods has been poorly studied, the aim of this work was to evaluate and compare the extraction potential of two different microextraction techniques based on MEPS and μ-SPEed® combined with UHPLC-PDA analysis in order to investigate the most suitable procedure for extracting polyphenols from baby food products. Important parameters that may affect the extraction efficiency, such as the amount and type of sorbent and solvent, were investigated and optimized. As far as we know, this is the first time that these microextraction techniques are evaluated and applied for the extraction of polyphenols in baby food samples. Thus, this work represents a first approach to determine the nutritional quality of this kind of products.

2. Material and methods

2.1. Reagents, materials and standards

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (ACN), methanol (MeOH), 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.

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).

The eVol® X-change® syringe and the μ-SPEed® cartridges (silica C18, porous PS/DVB reversed phase (RP), non-porous PS/DVB reversed phase (RP-NP), PS/DVB cationic exchange (SCX) and PS/DVB anionic exchange (SAX)), were kindly offered by EPREP (Mulgrave, Victoria, Australia). For MEPS, the eVOL® hand-held automatic analytical syringe and the BIN (Barrel Insert and Needle) containing the sorbent materials (C2 (ethyl-silica), C8 (octyl-silica), C18 (octadecyl-silica), SIL (unmodified silica), M1 (mixed-mode C8-SCX), DVB/HLB (divinylbenzene hydrophilic-lipophilic balance), HyDRC, PEP (HyperSep retain polar enhanced polymer), R-CX (HyperSep retain cationic exchange), R-AX (HyperSep retain anionic exchange), PGC (HyperSepHypercarb porous graphitized carbon), and SCX (strong cationic exchange)) were purchased from SGE Analytical Science (SGE Europe Ltd., United Kingdom).

2.2. Preparation of standard solutions

Individual stock standard solutions (1000 μg mL^{-1}) were prepared in MeOH and stored at −20 °C in darkness. A multicomponent standard solution of 20 μg mL^{-1} was prepared by dilution of each primary standard solution with MeOH and was used for optimization of the extraction conditions. For validation studies, working standard solutions containing the target analytes at different concentration levels were prepared daily by dilution of the individual stock solutions with MeOH. The target polyphenols were selected based on their importance and relevance on food quality, including the major classes (flavonoids and non-flavonoids).

2.3. Baby food samples

Four different commercial pureed baby foods: banana, apple, multifruits with cereals, and chicken, beef and vegetables, were purchased from a local pharmacy in Funchal, Portugal. Their declared composition according to their labels is given in Table 1SM (Supplementary Material). In order to obtain a liquid extract, the samples were subjected to a maceration process. For this purpose, 50 g of sample were mixed with 50 mL of MeOH:H_2O containing 0.1% FA (95:5 v/v), the mixture was kept in maceration for 24 h in darkness and then filtered under vacuum. The sample extracts were stored at 4 °C until analysis. Before extraction, the sample extracts were adjusted to pH 2.0, and all samples were extracted and analyzed in triplicate.

2.4. Extraction procedures

Two different microextraction techniques, MEPS and μ-SPEed®, were tested and compared in order to evaluate their ability to extract polyphenols from baby foods. In order to obtain the highest extraction efficiency, different parameters such as the chemical nature of the sorbent material, the number of extraction cycles, the pH, the elution solvent and the elution volume were optimized in both techniques.

2.4.1. Microextraction by packed sorbent (MEPS)

The MEPS procedure was performed using an eVol® hand-held automatic analytical syringe (500 μL) fitted with a BIN containing 4 mg of sorbent material and was used to draw and discharge samples and solutions through the BIN, according to Perestrelo, Silva, and Câmara (2015). Since the sorbent selection is an important factor to achieve acceptable clean-up and recoveries, twelve different MEPS sorbent materials: C2, C8, C18, SIL, M1, DVB/HLB, HyDRC, PEP, R-CX, R-AX, PGC and SCX were tested and compared in order to select the best one for the target analytes. To evaluate the number of extraction cycles

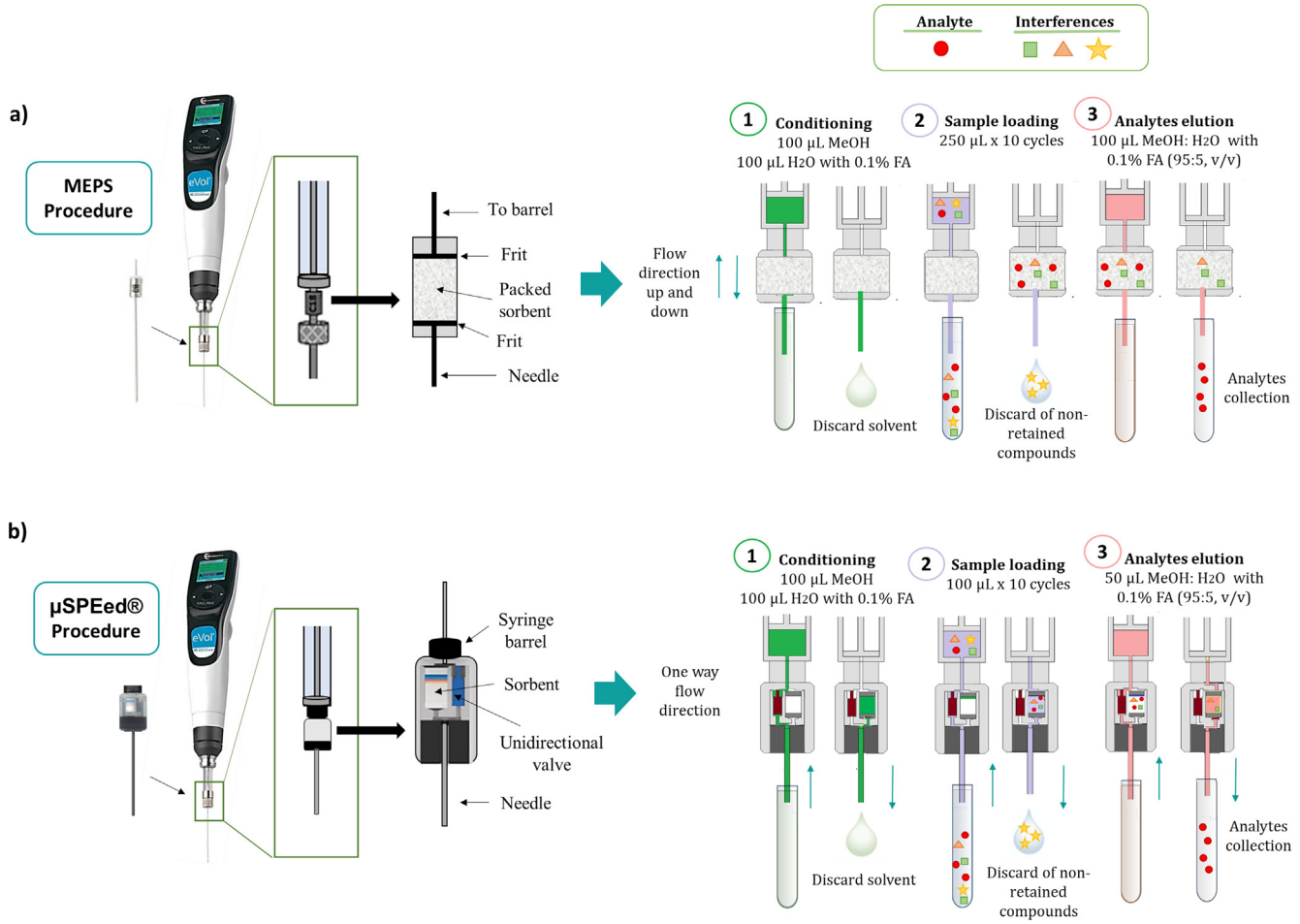

Fig. 1. Graphic representation of the optimized sample treatment procedures using MEPS (a) and µ-SPEed® (b) techniques.

(extract-discard) and sample volume, aliquots of 100, 250 and 500 µL of the multicomponent standard solution were pumped up and down 3, 5 and 10 times. In order to select a suitable extraction solvent, several solvent systems such as water, ACN and MeOH in different combinations were tested. In addition, different elution volumes (100, 150 and 200 µL) were also evaluated. Finally, the effect of pH in the extraction efficiency was studied within the range 2.0 to 10.0. All optimization procedures were carried out in triplicate. In all assays, the aspiration flow rate was automatically set to about 20 µL s^{-1} to prevent cavitation; this way the contact time and the extraction efficiency between the analyte and the sorbent are increased.

MEPS experiments were performed using 4 mg of DVB/HLB sorbent, previously selected as the best sorbent to isolate the target analytes in the optimization step. Fig. 1a depicts the MEPS procedure carried out under the optimized conditions. First the sorbent was conditioned with 100 µL of MeOH followed by 100 µL of H_2O containing 0.1% FA. Then, 250 µL of the sample extract (pH 2.0) were drawn through the syringe up and down 10 times at a flow rate of about 20 µL s^{-1}. No washing step was performed, so the analytes were directly eluted with 100 µL of MeOH:H_2O 0.1% FA (95:5, v/v) into a vial for subsequent analysis in the UHPLC-PDA system. Between extractions, the sorbent was rinsed with 250 µL of MeOH followed by 250 µL of H_2O containing 0.1% FA in order to avoid memory effects (carry-over) and also to act as conditioning step before the next extraction.

2.4.2. µ-SPEed®

The µ-SPEed® procedure was performed with an electronic eVol® X-change® hand-held automatic syringe (100 µL). First, for the optimization of the µ-SPEed® procedure, the extraction efficiency of the five available sorbents (silica C18, porous PS/DVB-RP, non-porous PS/DVB-RP, PS/DVB-SCX and PS/DVB-SAX) was evaluated and compared. To optimize the number of extraction cycles (extract-discard) and sample volume, aliquots of 25, 50 and 100 µL of the multicomponent standard solution were pumped up and down 3, 5 and 10 times. In order to obtain the highest extraction efficiency for the target analytes, different elution solvents, such as H_2O, ACN and MeOH, in different combinations were investigated. Several elution volumes (25, 50 and 100 µL) were also tested. Finally, the effect of pH in the extraction efficiency was assayed within the range 2.0 to 10.0. All optimization procedures were carried out in triplicate. In all µ-SPEed® assays the aspiration flow rate was also automatically set to about 20 µL s^{-1} to prevent cavitation as in MEPS.

µ-SPEed® experiments were performed using the porous PS/DVB-RP sorbent, previously selected as the best sorbent to extract the target analytes in the optimization step. Fig. 1b shows the µ-SPEed® procedure performed under the optimized conditions. Prior to each extraction, the sorbent was first conditioned with 100 µL of MeOH followed by 100 µL of H_2O containing 0.1% FA. Then, 100 µL of the sample extract (pH 2) were drawn through the syringe up and down 10 times. No washing step was performed, and the analytes were directly eluted with 50 µL of MeOH:H_2O containing 0.1% FA (95:5, v/v) into a vial for subsequent analysis in the UHPLC-PDA system. Between each extraction, the sorbent was rinsed with 2 × 100 µL of MeOH followed by 100 µL of H_2O containing 0.1% FA to avoid memory effects (carry-over) and to act as conditioning step before the next extraction.

2.5. UHPLC-PDA analysis and operating conditions

The separation and quantification of polyphenols was conducted 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 using 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 °C. A binary mobile phase with a gradient program was used, combining solvent A (H_2O 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 the samples were kept at 20 °C during the analysis. The UV detection wavelength was set to the maximum of absorbance for the target analytes. The identification of the polyphenols was based on the comparison of retention times (RT) and PDA spectra of their peaks in samples with those obtained using pure standards.

2.6. Analytical method validation

Both microextraction methodologies were properly validated in terms of selectivity, linear dynamic range (LDR), limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precision and accuracy.

Selectivity was assessed by the absence of interfering chromatographic peaks at the RT of the target analytes. LDR was evaluated at six concentration levels on standard solutions prepared and analyzed using the described extraction procedures. The concentration ranges were selected according to the sensitivity of the UHPLC-PDA system towards each target analyte, as well as the amount expected in samples. 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. The LODs (the lowest analyte concentration that produces a response detectable above the noise level of the system) and LOQs (the lowest level of analyte that can be accurately and precisely measured) of each compound were calculated considering the concentration that produced a signal-to noise ratio (S/N) equal or higher than 3 and 10, respectively.

The accuracy, expressed as recovery percentage (%), was assessed by spiking the sample extracts obtained from the chicken, beef and vegetables baby food in triplicate at three concentration levels (low, medium and high) and subjecting them to the extraction procedures described above. The recovery values were determined by comparison of the areas of the spiked samples with the areas of simulated samples (sample extracts spiked at the same concentration levels but at the end of the extraction process, evaporated to dryness and reconstituted in MeOH). 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 were performed on the same day, by the same analyst to obtain intra-day precision. For inter-day precision, six replicated of each level were analyzed daily through three different days ($n = 18$).

For quantification purposes, the matrix effect (ME) was evaluated according to the following equation:

$$ME(\%) = (B/A) \times 100$$

where A is the mean peak area of the analyte in the standard solution and B is the mean peak area of the analyte in the spiked sample extracts after extraction. The samples were classified according to their composition, therefore the multi-fruits with cereals baby food was chosen as representative sample of fruit-based baby foods, since it had banana and apple in its composition. Thus, to estimate the ME six replicates of standard solution at the medium concentration level were injected into the UHPLC-PDA, and six replicates of blank sample extracts obtained from the multi-fruits with cereals and the chicken, beef and vegetables baby foods were prepared using both optimized extraction procedures. At the end of the extraction processes the extracts obtained were spiked with the analytes at the medium level concentration, evaporated to dryness, reconstituted in MeOH and injected into the UHPLC-PDA system.

3. Results and discussion

3.1. MEPS analysis

Several parameters were evaluated to establish the optimal conditions for the MEPS procedure including sorbent nature, number of extraction cycles, elution solvent, elution volume, sample volume and pH. All assays were performed in triplicate for each optimized extraction parameter and the extraction efficiency was determined by the average total peak area response observed on the UHPLC-PDA and % RSD.

Twelve different MEPS sorbents were tested under the same extraction conditions (3 extraction cycles using 250 µL of standard solution at pH 2.0 and desorption with 100 µL of MeOH:H_2O containing 0.1% FA (95:5 v/v)), the results are presented in Fig. 2. As it can be observed (Fig. 2A), polymeric sorbents (DVB/HLB, HyDRC, PEP, R-CX and R-AX) clearly exhibited better extraction efficiency than the silica-based sorbents (C2, C8, C18, SIL, M1 and SCX) and the carbon-based sorbent (PGC). The DVB/HLB sorbent was selected as the most adequate since it provided, on average, the best repeatability and the highest chromatographic response under the tested conditions.

In MEPS the number of extraction cycles influences the extraction efficiency of the analytes. The sample can be drawn up and down through the syringe once or several times (cycles). There are two ways to performed the multiple extraction cycles: draw-eject (discard in the same vial of the sample) or extract-discard (discard in a waste vial). Extract-discard mode was chosen since it provides better responses and does not induce high mechanical stress in the syringe plunger, expanding the MEPS syringe lifetime. The effect of the number of extraction cycles (3, 5 and 10 extract-discard) on the extraction efficiency is illustrated in Fig. 2B. The extraction efficiency increased when the number of cycles increased, therefore 10 cycles were selected since they provided the highest efficiency. The sample volume was also evaluated, 500 µL of sample provided the highest chromatographic area. However, the increase in the area response was not proportional while increasing the sample volume (Fig. 2D), so to avoid sorbent clogging and low recoveries, an intermediate volume of 250 µL was finally selected for the MEPS procedure. The impact of sample pH was studied within the range 2.0–10.0. Results showed (Fig. 2C) that pH 2.0 enhanced the adsorption of the target polyphenols, while at pH 7.0 and 10.0 the extraction efficiency was significantly lower. Therefore, a pH adjustment (2.0) was done through all the experimental analysis with MEPS. For desorbing conditions, the solvent and the elution volume were investigated to ensure effective elution of the analytes from the sorbent. First, different combinations of MeOH and ACN with acidified water were evaluated to optimize the elution of the target analytes by MEPS. Fig. 2E shows the UHPLC-PDA average response for the target analytes using the different elution solvents. Generally, the analyte response increased as the organic solvent percentage increased. MeOH:H_2O containing 0.1% FA (95:5 v/v) was slightly better than ACN:H_2O containing 0.1% FA (95:5 v/v), and since MeOH was the solvent used on the gradient mobile phase system, which can contribute to minimize the matrix effect and to increase chromatographic resolution, it was selected as the best elution solvent. Finally, different elution volumes (100, 150 and 200 µL) of MeOH:Water 0.1% FA (95:5 v/v) were studied. It was observed that the analyte response decreased when the

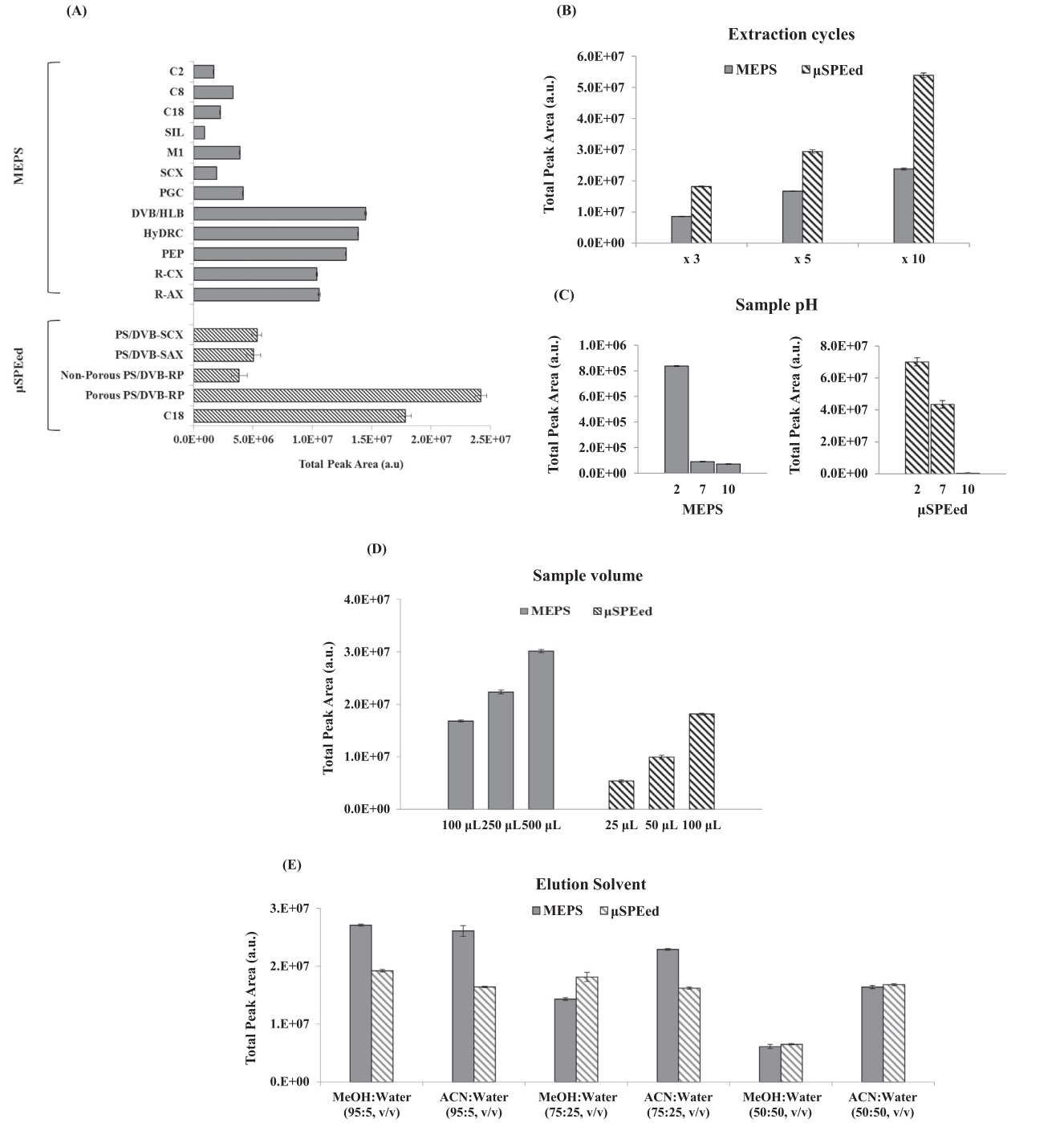

Fig. 2. Effect of the (A) *sorbent nature* (3 extraction cycles × 250 µL standard solution pH 2, desorption 100 µL MeOH:H_2O containing 0.1% FA (95:5 v/v)) and µ-SPEed® (3 extraction cycles × 100 µL standard solution pH 2, desorption 100 µL MeOH: H_2O containing 0.1% FA (95:5 v/v)); (B) number of extraction cycles; (C) pH; (D) sample volume; and (E) elution solvent; on the extraction efficiency of the selected polyphenols by MEPS and µ-SPEed®. Values expressed as mean ± standard deviation (n = 3).

elution volume increased, because high elution volumes produced a dilution effect. Therefore, a volume of 100 µL was selected for desorption of analytes.

Overall, based on this data, the best MEPS experimental conditions for the analysis of the target polyphenols were: DVB/HLB sorbent, 10 draw-eject cycles (10 × 250 µL of sample at pH 2.0) and desorption with 100 µL of MeOH:H_2O containing 0.1% FA (95:5 v/v), as it is shown in Fig. 1a.

To demonstrate the feasibility of the newly proposed analytical strategy and evaluate its practical applicability for quantification of polyphenols in baby food samples, the method was validated in terms of selectivity, linearity, LOD, LOQ, intra/inter-day precision and accuracy. The validation parameters are shown in Tables 1 and 2. Method selectivity was demonstrated since no interfering peaks were found at the RT of the target analytes at their quantification wavelengths (Table 1).

The polyphenols were identified by comparing RT and ultraviolet absorption spectra. The representative chromatogram of a standard mixture measured at 255 nm (wavelength at which all analytes absorbed) with the chromatographic method is depicted in Fig. 1SM (Supplementary Material).

Table 1
Comparison of the analytical figures of merit of the proposed MEPS/UHPLC-PDA and μSPEed/UHPLC-PDA methodologies.

Phenolic compounds	RT[a] (min)	Wavelength (nm)	Microextraction technique	LDR[b] (μg mL^{-1})	Equation	[c]R^2	LOD[d] (μg kg^{-1})	LOQ[e] (μg kg^{-1})
Gallic acid	2.1	271	MEPS	0.25–2.00	y = 8956x − 287	0.994	12.8	42.5
			μSPEed		y = 15132x − 1885	0.997	2.1	6.9
Protocatechuic acid	3.1	259	MEPS	0.10–1.50	y = 8039x + 657	0.998	1.0	3.3
			μSPEed		y = 14128x + 180	0.993	2.3	7.6
Chlorogenic acid	4.1	326	MEPS	0.03–0.75	y = 11933x + 620	0.995	1.7	5.6
			μSPEed		y = 13275x + 438	0.990	2.5	8.3
4-Hydroxybenzoic acid	4.5	255	MEPS	0.10–2.00	y = 57490x + 10472	0.992	5.3	17.6
			μSPEed		y = 154525x − 5625	0.999	5.1	17.0
Epicatechin	5.0	278	MEPS	0.05–1.50	y = 2176x + 954	0.995	7.1	23.8
			μSPEed		y = 4193x − 135	0.993	3.8	12.6
p-Coumaric acid	6.1	309	MEPS	0.03–0.75	y = 13705x + 93	0.992	2.1	7.2
			μSPEed		y = 21379x − 465	0.996	1.4	4.6
Ferulic acid	6.5	323	MEPS	0.01–0.95	y = 7784x + 650	0.993	1.6	5.4
			μSPEed		y = 11452x + 472	0.992	1.6	5.2
Rutin	8.0	354	MEPS	0.05–2.00	y = 2752x + 653	0.992	9.6	31.9
			μSPEed		y = 4185x − 206	0.984	2.5	8.4
Resveratrol	8.3	305	MEPS	0.03–0.75	y = 16395x + 120	0.997	1.8	5.9
			μSPEed		y = 16734x + 212	0.992	1.4	4.7
Myricetin	9.1	372	MEPS	0.03–0.75	y = 3543x − 49	0.992	3.3	11.2
			μSPEed		y = 3823x − 311	0.998	2.8	9.2
Naringenin	10.1	289	MEPS	0.05–1.50	y = 6147x + 1062	0.992	10.0	33.3
			μSPEed		y = 8690x + 333	0.992	7.8	26.1
Kaempferol	10.9	363	MEPS	0.05–1.50	y = 7623x + 1843	0.985	7.4	24.6
			μSPEed		y = 7030x + 3096	0.980	13.6	45.2

[a] RT Retention time
[b] LDR linear dynamic range
[c] R^2 linear correlation coefficient
[d] LOD, limit of detection, calculated for a S/N of 3.
[e] LOQ, limit of quantification, calculated for a S/N of 10.

As shown in Table 1, the method showed good linearity (with $R^2 \geq 0.985$), low LODs (ranging from 1.0 to 12.8 μg kg^{-1}) and LOQs (ranging from 3.3 to 42.5 μg kg^{-1}) and good reproducibility (RSD < 5%). To evaluate the accuracy of the method, the sample extract obtained from the chicken, beef and vegetables baby food was selected to carry out the recovery studies. For this purpose, the sample extract was spiked at three concentration levels with a known amount of each target polyphenol. The spiking levels used for the accuracy and precision studies are summarized in Table 2.

The accuracy was determined according to the procedure explained in Section 2.6. In each set of experiments, the sample extracts were spiked in triplicate at three concentration levels, and a simulated sample for each level was prepared in the same way but spiked with the analytes at the end of the extraction procedure, evaporated to dryness and reconstituted in MeOH. The recoveries were calculated by comparison of the areas of the samples with the areas of their corresponding simulated sample. The average recovery of the polyphenols is listed in Table 2. Low recoveries values were obtained for the majority of the phenolic acids (4-hydroxybenzoic acid, chlorogenic acid, ferulic acid and gallic acid) ranging between 47 and 63 %. On the other hand, the method accuracy was adequate for the rest of the compounds, since the mean recovery values ranged from 70 to 98%. Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility, expressed as % RSD. The intra-day repeatability was calculated by analyzing in the same day six replicates of chicken, beef and vegetables pureed sample extract spiked with the target analytes at three concentration levels, while inter-day reproducibility was determined by analyzing the spiked samples within a 3-day period. Satisfactory results were achieved with RSD values lower than 4% for intra-day precision, and lower than 5% for inter-day precision (Table 2), indicating the strong stability of the developed method.

3.2. μ-SPEed® analysis

The optimization of the μ-SPEed® procedure was performed in the same way than the optimization of the MEPS procedure, through the evaluation of the same extraction parameters (sorbent nature, number of extraction cycles, elution solvent, elution volume, sample volume and pH).

Five different μ-SPEed® sorbents were tested under the same extraction conditions: 3 extraction cycles × 100 μL of standard solution at pH 2.0 and desorption with 100 μL of MeOH:H_2O containing 0.1% FA (95:5 v/v). Porous PS/DVB-RP and C18 sorbents provided the best extraction efficiency and reproducibility (Fig. 2). However, the porous PS/DVB-RP sorbent showed higher performance for all the target analytes. On the other hand, the non-porous sorbents PS/DVB-RP-NP, PS/DVB-SAX and PS/DVB-SCX provided lower and unsatisfactory retention of the target polyphenols. These results can be justified by the type of interactions involved and the superficial area of interaction between the sorbent particles and the target analytes. The higher surface area of the porous reversed-phase or silica sorbents allows increasing the binding capacity over the non-porous and ion-exchangers sorbents. In addition, the bigger pore size of the porous PS/DVB-RP sorbent (300 Å *vs* 120 Å of C18 sorbent) corresponds to a higher superficial area of interaction and consequently to a better retention of the polyphenols. For this reason, porous polymeric sorbents have higher loading capacities than their silica counterparts (Pereira, Gonçalves, Alves, & Câmara, 2013; Porto-Figueira et al., 2015). The porous PS/DVB-RP sorbent was selected as the best sorbent since it allowed the higher extraction efficiency and reproducibility under the tested conditions.

Table 2
Relative recovery and precision studies performed on real samples, chicken, beef and vegetable-based baby foods, spiked with the target polyphenols at low, medium and high levels, using the MEPS and μSPEed extraction procedures.

Phenolic compounds	Spiked levels (μg mL^{-1})	MEPS			μSPEed		
		Recovery (%)	Intra-day precision (RSD %)	Inter-day precision (RSD %)	Recovery (%)	Intra-day precision (RSD %)	Inter-day precision (RSD %)
Gallic acid	0.25[a]	67.3	2.3	4.2	67.3	2.8	4.5
	0.50[b]	60.4	2.2	4.5	68.7	2.2	4.9
	1.00[c]	61.1	2.9	4.8	65.4	3.0	3.0
	AVG[4]	**63 ± 3.7**			**67 ± 1.7**		
Protocatechuic acid	0.20[a]	73.9	3.3	3.1	81.0	1.6	3.4
	0.60[b]	80.0	3.1	4.1	90.0	1.4	1.8
	1.50[c]	78.2	2.8	3.8	83.9	1.0	2.1
	AVG	**77 ± 3.1**			**85 ± 4.6**		
Chlorogenic acid	0.03[a]	52.4	2.4	4.1	86.5	2.6	3.2
	0.13[b]	55.8	1.8	3.1	74.4	2.2	2.3
	0.38[c]	50.0	2.1	4.2	71.6	1.5	1.8
	AVG	**53 ± 2.9**			**77 ± 7.9**		
4-Hydroxybenzoic acid	0.10[a]	47.5	0.8	1.0	51.2	1.5	3.3
	0.50[b]	46.7	1.0	1.5	53.8	1.9	2.0
	1.00[c]	48.4	1.7	2.2	52.6	1.7	2.3
	AVG	**47.5 ± 0.9**			**52 ± 1.3**		
Epicatechin	0.05[a]	73.9	3.2	3.3	92.7	2.3	3.7
	0.25[b]	79.4	3.3	4.3	97.9	1.7	3.2
	0.75[c]	89.3	2.7	4.5	89.8	2.1	3.8
	AVG	**81 ± 7.8**			**93 ± 4.1**		
p-Coumaric acid	0.05[a]	67.7	2.0	2.5	75.9	2.7	4.8
	0.13[b]	69.1	2.4	2.6	63.0	1.8	2.8
	0.25[c]	75.3	2.2	2.4	61.3	1.1	1.8
	AVG	**71 ± 4.0**			**67 ± 8.0**		
Ferulic acid	0.05[a]	57.4	1.0	1.8	90.4	1.1	1.9
	0.13[b]	66.6	1.7	2.2	81.5	1.7	3.4
	0.25[c]	63.8	2.5	1.6	87.4	1.2	2.9
	AVG	**63 ± 4.7**			**86 ± 4.5**		
Rutin	0.05[a]	74.8	1.6	1.9	85.7	2.1	2.8
	0.25[b]	66.6	2.0	2.3	72.4	2.6	4.7
	0.75[c]	69.4	2.8	3.0	72.0	1.4	2.8
	AVG	**70 ± 4.2**			**77 ± 7.8**		
Resveratrol	0.03[a]	74.2	2.6	2.8	81.8	2.4	1.7
	0.13[b]	87.9	2.8	3.1	87.2	1.1	1.3
	0.38[c]	88.0	2.3	2.7	83.6	2.1	5.2
	AVG	**83 ± 7.9**			**84 ± 2.7**		
Myricetin	0.03[a]	81.3	1.5	3.1	94.5	2.2	4.8
	0.13[b]	71.2	2.3	3.5	97.4	1.8	2.2
	0.38[c]	84.8	2.1	3.9	95.7	2.3	4.2
	AVG	**79 ± 7.1**			**96 ± 1.5**		
Naringenin	0.05[a]	95.6	1.0	3.5	98.8	1.4	4.1
	0.25[b]	91.4	1.3	3.3	90.7	1.2	4.4
	0.75[c]	91.7	1.9	3.8	85.0	1.7	2.2
	AVG	**93 ± 2.3**			**91 ± 6.9**		
Kaempferol	0.05[a]	100.1	2.2	4.2	93.3	1.8	3.1
	0.25[b]	96.4	2.7	3.7	92.1	2.8	4.6
	0.75[c]	98.2	2.4	4.5	102.7	1.2	1.5
	AVG[d]	**98 ± 1.9**			**97 ± 5.8**		

[a] Low concentration level.
[b] Medium concentration level.
[c] High concentration level.
[d] AVG – average value ± sd.

In μ-SPEed®, the number of extraction cycles also influences the retention of the analytes. As in MEPS, the multiple extraction cycles can be performed by draw-eject or extract-discard mode, the last option was selected in the present work. Fig. 2B shows the effect of the number of extraction cycles (3, 5 and 10 extract-discard) on the extraction efficiency. The retention of the target analytes was higher as the number of cycles increased. Therefore, 10 extraction cycles were selected since they provided the best performance. Different sample volumes (25, 50 and 100 μL) were tested, and best results were achieved with 100 μL (Fig. 2D). The sample pH was also evaluated within the range 2.0–10.0. As in the MEPS procedure, pH 2.0 enhanced the adsorption of the target compounds (Fig. 2C), thus a pH adjustment was carried out through all the experimental analysis with μ-SPEed®. In order to evaluate the effect of the solvent on the extraction efficiency, different combinations of MeOH and ACN with acidified water were evaluated and compared. As it can be observed in Fig. 2E, best extraction was achieved using MeOH:H_2O containing 0.1% FA (95:5 v/v) to elute the target analytes. This result is consistent with the one previously described in the MEPS

Table 3
Occurrence of the target polyphenols in different baby foods analysed by MEPS/UHPLC-PDA and μSPEed/UHPLC-PDA methodologies.

Phenolic compounds	Microextraction technique	Banana		Apple		Multi-fruits with cereals		Chicken, beef and vegetables	
		Concentration (μg kg^{-1})	RSD(%)	Concentration (μg kg^{-1})	RSD(%)	Concentration (μg kg^{-1})	RSD(%)	Concentration (μg kg^{-1})	RSD(%)
Gallic acid	MEPS	99	5.3	71	2.4	133	4.6	112	1.8
	μSPEed	138	7.3	169	3.8	154	2.0	125	1.1
Protocatechuic acid	MEPS	48	6.8	48	5.2	-[b]	–	–	–
	μSPEed	109	2.8	50	3.9	–	–	–	–
Chlorogenic acid	MEPS	–	–	815	2.2	569	2.6	194	3.1
	μSPEed	–	–	1790	1.4	1353	2.0	284	4.1
4-Hydroxybenzoic acid	MEPS	31.5	1.7	–	–	43	3.1	31	5.1
	μSPEed	37	4.1	–	–	44.7	1.9	30	4.0
Epicatechin	MEPS	153	3.7	469	1.8	293	3.3	–	–
	μSPEed	294	2.9	565	2.4	416	3.6	–	–
p-Coumaric acid	MEPS	67	2.6	32.6	2.3	64	3.0	65	3.3
	μSPEed	63	5.5	31.0	2.6	60.3	1.4	71.5	1.3
Ferulic acid	MEPS	236	2.5	–	–	365	2.2	112	3.7
	μSPEed	307	3.8	–	–	409	1.6	165	1.3
Rutin	MEPS	340	3.4	169	2.5	360	2.3	< LOQ[a]	–
	μSPEed	387	3.2	192	4.3	367	1.7	36.3	1.3
Resveratrol	MEPS	–	–	–	–	–	–	–	–
	μSPEed	–	–	–	–	–	–	–	–
Myricetin	MEPS	108	5.1	52	2.0	98	2.2	< LOQ	–
	μSPEed	179	3.1	74	2.9	161	3.3	< LOQ	–
Naringenin	MEPS	128	3.2	61	3.0	285	5.7	148	5.4
	μSPEed	104	4.3	65	4.9	307	4.4	147	1.6
Kaempferol	MEPS	< LOQ	–	< LOQ	–	< LOQ	–	110	6.4
	μSPEed	< LOQ	–	52	4.3	< LOQ	–	71	3.0

[a] < LOQ: lower than limit of quantification.
[b] –: not detected.

optimization. Finally, different elution volumes (25, 50 and 100 μL) of MeOH:Water 0.1% FA (95:5 v/v) were studied. The volume of 25 μL was too small for its injection in the UHPLC-PDA, so no data were obtained for this volume, and 50 μL of MeOH:H_2O containing 0.1% FA (95:5 v/v) were selected as the best elution conditions.

Overall, the best μ-SPEed® experimental conditions for the analysis of the target polyphenols were: porous PS/DVB-RP sorbent, 10 draw-eject cycles (10 × 100 μL of sample at pH 2.0) and desorption with 50 μL of MeOH:H_2O containing 0.1% FA (95:5 v/v), as it is shown in Fig. 1b.

Under the optimized conditions, the newly proposed analytical method for quantification of polyphenols in baby food samples by μ-SPEed® was validated in terms of selectivity, linearity, LOD, LOQ, intra/inter-day precision and accuracy. The validation parameters are shown in Tables 1 and 2.

Method selectivity was assessed by the absence of any interfering peak at the expected RT for the maximum absorption quantification wavelengths of the target analytes. The UHPLC-PDA system gave linear response over the studied range of concentrations and the least-squares linear regression analysis of the data provided excellent correlation coefficient (R^2) values above 0.990 for all the polyphenols, except rutin and kaempferol (0.984 and 0.980, respectively) (Table 1). The method showed LODs ranging between 1.37 (*p*-coumaric acid) and 13.57 (kaempferol) μg kg^{-1} and LOQs between 4.57 (*p*-coumaric acid) and 45.23 (kaempferol) μg kg^{-1} (Table 1). Recovery studies were carried out in the same way than for the MEPS procedure, spiking the extracts obtained from the chicken, beef and vegetables pureed sample in triplicate at three concentration levels and using a simulated sample for each level in order to compare the areas and calculate the recovery percentage. Satisfactory results were found in all the concentration levels for most of the analytes, ranging from 77 (rutin) to 97% (kaempferol) (Table 2). Only low recovery values were found for 4-hydroxybenzoic acid (52%), *p*-coumaric acid (67%) and gallic acid (67%). The precision, evaluated in terms of intra-day repeatability and inter-day reproducibility, expressed as % RSD, was also very good, with RSD values lower than 5% in both cases (Table 2).

Overall, the combination of μ-SPEed® with UHPLC-PDA analysis proved to be a very efficient strategy for the extraction and quantification of the selected polyphenols, revealing excellent performance in terms of linearity, sensitivity, precision and accuracy.

3.3. Evaluation of MEPS and μ-SPEed® for the analysis of polyphenols

In order to evaluate the efficiency of the target microextraction techniques, the analytical performance of both proposed techniques, MEPS and μSPEed®, were compared (Tables 1 and 2).

Both techniques are very similar adsorption-based methods. However, best performance was achieved with the μ-SPEed® procedure, since it provided, in general, higher recovery values of the target analytes and very low LODs and LOQs. In addition, it uses fewer amounts of solvents, sample and time than MEPS. These results can be justified because the μ-SPEed® configuration has several advantages over the MEPS procedure, being one of the most remarkable the direct flow through the sorbent bed (Fig. 1). The existence of a pressure-drived one-way check valve allows an ultra-low dead volume connection, and aspiration, which can only be achieved by means of vacuum when the plunger is pulled back, does not have to pass the bed but bypasses the sorbent. This enables to use smaller sorbent particles (< 3 μm, instead of the 50 μm used in MEPS) improving the extraction/elution efficiency. In addition, during elution, thanks to the one-way direction valve, the analytes retained in the sorbent are not disturbed by the solvent aspiration, unlike MEPS (Fig. 1).

Table 4
Comparison of matrix effect of MEPS and μSPEed procedures.

Phenolic compounds	Chicken, beef and vegetables baby food matrix effect (%)		Fruit-based baby food matrix effect (%)	
	MEPS	μSPEed	MEPS	μSPEed
Gallic acid	32	46	50	42
Protocatechuic acid	41	17	40	23
Chlorogenic acid	22	63	23	44
4-Hydroxybenzoic acid	37	50	16	16
Epicatechin	21	17	28	16
p-Coumaric acid	90	113	27	24
Ferulic acid	122	100	32	31
Rutin	93	77	47	88
Resveratrol	82	87	23	23
Myricetin	122	128	28	68
Naringenin	60	64	44	48
Kaempferol	45	39	89	100

Therefore, it was concluded that best results were achieved with the proposed μSPEed® procedure, since it proved to be a more sensitive extraction technique with excellent performance (linearity, sensitivity, precision and accuracy) showing great potential for the determination of polyphenols in baby food samples.

3.4. Application of MEPS and μ-SPEed® on baby food samples

The occurrence of the target polyphenols in commercial pureed baby foods: banana, apple, multi-fruits with cereals, and chicken, beef and vegetables, intended for infants and young children commercially available in the Portuguese markets was investigated. Table 3 summarizes the results obtained for all the samples analyzed.

The areas of the compounds that were clearly recognized by their PDA spectrum and RT were extracted, and for quantification purposes, they were subjected to correction using the ME calculated for the fruit-based and the chicken, beef and vegetables baby food samples (Table 4).

As it can be observed (Table 3), the profile and concentrations of the analytes varied in the different matrices, and not all were detected in each sample. Some polyphenols were detected at concentrations levels lower than their LOQ and could not be quantified. Regarding the total concentration of the target polyphenols, the multi-fruits with cereals baby food was the sample with the highest amount, followed by the apple pureed sample, while the chicken, beef and vegetables baby food showed the lowest concentration of polyphenols. The most abundant polyphenols quantified in the apple pureed sample were chlorogenic acid, epicatechin, rutin and gallic acid, while ferulic acid, rutin, epicatechin, myricetin and gallic acid were the main polyphenols quantified in the banana pureed sample. These results are in agreement with the fact that these compounds are the main polyphenols found in apple and banana fruits according to previous reports (Hjelmeland, Wylie, & Ebeler, 2016; Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, & Lewandowski, 2014; Lucci, Saurina, & Núñez, 2017; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Singh, Singh, Kaur, & Singh, 2016). Rutin is a flavonoid especially found in citrus fruits such as orange and lemon. Therefore, the high occurrence of rutin in the banana baby food could be due to the addition of concentrate lemon juice in its composition (Table 1SM). Moreover, protocatechuic acid, *p*-coumaric acid and myricetin, a popular polyphenol because of its bioactive properties against cancer and cardiovascular diseases (Godse, Mohan, Kasture, & Kasture, 2010; Sun et al., 2012), were also present at moderate concentrations in the banana and apple pureed samples (Table 3). In the multi-fruits with cereals baby food the 96% of its composition were fruits, among them apple and banana (Table 1SM). Therefore, according to the previous samples, chlorogenic acid, epicatechin, rutin, myricetin and gallic acid were also the main polyphenols found in this sample, in addition to ferulic acid which was also present since is the most representative polyphenol in cereals (Lucci et al., 2017; Manach et al., 2004) (Table 3). Naringenin is a flavanone with high chemopreventive and therapeutic potential which is usually abundant in citrus fruits (Lucci et al., 2017; Manach et al., 2004; Mir & Tiku, 2015). Therefore, its significant occurrence in the multi-fruits with cereals baby food (Table 3) is probably due the presence of orange juice in its composition (Table 1SM). Naringenin was also detected at lower level in the other samples, probably as a result of the addition of lemon juice to their composition. The chicken, beef and vegetables pureed sample exhibited the lowest amount of polyphenols. Chlorogenic acid, ferulic acid, gallic acid and naringenin were the main polyphenols found in this sample (Table 3). The occurrence of ferulic acid was probably due to the presence of rice and corn starch in its composition (Table 1SM), since it is the most abundant polyphenol in cereals (Lucci et al., 2017; Manach et al., 2004), while it has been reported that chlorogenic acid is the main polyphenol present in potatoes and carrots (Deußer, Guignard, Hoffmann, & Evers, 2012; Furrer, Cladis, Kurilich, Manoharan, & Ferruzzi, 2017; Ma et al., 2013). In addition, the presence of tomatoes and lemon juice as ingredients probably contribute to the levels of naringenin found in this sample. Moreover, *p*-coumaric acid, kaempferol, 4-hydroxybenzoic acid and rutin were also quantified at lower levels, which are characteristic polyphenols present in vegetables such as onions, potatoes and tomatoes (Deußer et al., 2012; Furrer et al., 2017; Lucci et al., 2017; Manach et al., 2004), which were ingredients of this baby food sample (Table 1SM).

4. Conclusions

Two high-throughput microextraction techniques, MEPS and μ-SPEed®, combined with UHPLC-PDA analysis, were evaluated and compared to determine their performance for the simultaneous quantification of 12 polyphenols in baby food products, involving minimal sample pre-treatment and solvent usage. To enable an effective comparison, selectivity, LDR, LODs, LOQs, accuracy, precision and extraction yields, were determined and discussed for both techniques. The results obtained showed that both methodologies present great advantages including, high selectivity and extraction efficiency in very short extraction time with minimal solvent consumption and fast sample throughput, being more environmentally friendly and easier to perform than classic extraction techniques. However, μ-SPEed® showed higher performance than MEPS. The analytical procedures developed were applied for the determination of polyphenols in different baby food samples, and the results obtained allowed characterizing the abundance of the selected polyphenols in several baby food products. Thus, this work represents a first approach to evaluate and improve the knowledge of the nutritional quality of this kind of products. In addition, these methodologies can potentially be extended to other extraction media, matrices and analytes.

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Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.04.038.

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