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Optimization of Sample Preparation Procedure for Determination of Fat-Soluble Vitamins in Milk and Infant Food by HPLC Technique

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Abstract: Background: The analysis of vitamins in baby food is a challenging task given the complexity of the food matrix, vitamin stability, and strict regulations of the European Union regarding permissible deviations from declared values. Vitamins in food exist in different concentrations and forms and have different stabilities, and it is not straightforward to prepare samples for reliable analysis using the same procedure. Therefore, significant attention has been devoted to optimizing sample preparation in the analysis of vitamins. Methods: This study aims to determine which of the seven applied extraction methods is the most efficient for the simultaneous extraction and determination of A, D, E, and K vitamins in milk and baby food using high-performance liquid chromatography (HPLC). Different samples of baby food were prepared in seven different ways based on four methods (saponification, enzymatic hydrolysis, solvent extraction, and solid-phase extraction). Results and Conclusion: The best preparation method proved to be solid-phase extraction with a C18 stationary phase, and HPLC with a UV-VIS detector was identified as a sufficiently sensitive technique for the identification and quantification of fat-soluble vitamins in milk and baby food.

Keywords: vitamins; baby food; extraction; HPLC

1. Introduction

Vitamins are essential compounds crucial for the normal functioning of the human body, facilitating numerous enzymatic and metabolic functions. They are categorized into two groups based on their solubility: fat-soluble vitamins A, D, E, and K, which are soluble in fats, and water-soluble vitamins C and B complexes [1–4]. Most vitamins cannot be synthesized by the body and must be obtained through dietary intake, with exceptions being vitamins D and K. Despite being required in small quantities, their deficiency or excessive intake can lead to severe health problems, particularly in sensitive age groups. Therefore, the diet for infants and young children must be well-balanced, containing sufficient vitamins and other nutrients to ensure proper growth and development. Vitamins differ from other nutrients (lipids, carbohydrates, proteins) as they do not have a structural role, nor do they produce energy. Instead, they participate in various metabolic processes as antioxidants, regulators, or coenzymes [5–9]. The solubility of vitamins is determined by their chemical composition. Water-soluble vitamins contain carbon, hydrogen, oxygen, nitrogen, sulfur, and cobalt (except for vitamin C), while fat-soluble vitamins contain only carbon, hydrogen, and oxygen atoms. Vitamins A and E are stable in both acidic and alkaline environments, vitamin D is unstable in acidic conditions but stable in alkaline surroundings, while vitamin K is unstable in both acidic and alkaline media [10,11]. The analysis of vitamins in baby food poses a challenging and complex procedure due to the food's content complexity, the chemical form of vitamins present, their

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stability, and European Union regulations on allowable deviations in food vitamins. Until today, the most frequently used technique for quantitative determination of vitamins in food samples is high-performance liquid chromatography (HPLC). However, due to variations in quantities and forms in which vitamins are present in food, the isolation and quantification of vitamins demand an analytical approach where sample preparation is the most crucial part of the procedure. Consequently, in recent years, significant attention has been directed towards optimizing sample preparation in vitamin analysis [12–15].

Vitamin A, also known as retinol, belongs to the retinoid group, compounds with a basic structure consisting of a trimethylated cyclohexane ring, polyene side chain, and a polar functional group at the terminal C atom. The terminal functional group can be hydroxyl (retinol), aldehyde (retinal), carboxyl (retinoic acid), or ester (retinyl ester). Retinol and its esters (acetate and palmitate) exhibit the most pronounced vitamin activity. Due to their predominantly hydrophobic nature, retinoids are insoluble in water but soluble in organic solvents such as fats, oils, hexane, diethyl ether, acetone, chloroform, ethanol, and methanol. The conjugated double bonds in the chain make retinoids unstable in the presence of light, oxidants, and heat, leading to oxidation or isomerization. The conjugated polyene chain is responsible for the strong absorption of UV-Vis radiation (325 – 380 nm). From the mid-1970s to the present, the analytical method for determining vitamin A in complex matrices such as blood serum and food is HPLC with UV-Vis detector or coupled LC-MS. Additionally, retinoids can be determined by spectrophotometric methods [16,17].

Vitamin D encompasses a group of secosteroids, a subclass of steroids where one bond in the B steroid ring is "broken." There are seven types of vitamin D, with only two being biologically active: vitamin D2 or ergocalciferol and vitamin D3 or cholecalciferol. Structurally, they differ only at two positions on the side chain; vitamin D2 has a double bond at the C-22 atom and an additional methyl group at the C-24 atom, which vitamin D3 lacks [18,19]. Vitamins D2 and D3 are insoluble in water but soluble in most organic solvents like ethanol, acetone, chloroform, etc. They are unstable in light and air, stable in basic conditions, and unstable in acidic conditions; in mildly acidic conditions, they isomerize to the 5,6-trans isomer of vitamin D and isotachysterol [18–20]. The most common technique for determining vitamins D2 and D3 is HPLC with a UV-Vis detector (λ = 265 nm).

Vitamin E refers to a group of tocopherols, compounds with a structure consisting of a chromanol ring attached to one to three methyl groups and an isoprenoid chain of 16 carbon atoms. Based on the saturation of the hydrocarbon chain, tocopherols are divided into tocopherols (saturated bonds) and tocotrienols (unsaturated bonds). There are four tocopherols and four tocotrienols in nature, denoted as α, β, γ, and δ compounds, based on the number and position of methyl groups on the ring. α-tocopherol (5, 7, 8-trimethyl tocopherol) is the biologically most active form of vitamin E. Tocopherols and tocotrienols are soluble in organic solvents but insoluble in water. They are unstable in light and easily oxidize in the air, forming biologically inactive quinones. They remain stable at high temperatures and in acidic and basic conditions in the absence of oxygen and light. Similar to vitamins A and D, the most common method for determining vitamin E is HPLC with a UV-Vis detector (λ = 292 – 296 nm), but sometimes other methods such as spectrophotometry, Raman, IR spectroscopy, and radioimmunoassay are used [21–24].

Vitamin K designates a group of several vitamins with a basic structure consisting of a naphthoquinone ring substituted with a methyl group at the C-2 position and a saturated/unsaturated hydrocarbon chain at the C-3 position. There are two active forms of vitamin K in nature: phylloquinone or vitamin K1 and menaquinones or vitamin K2. Menaquinones are a group of structural analogs that can contain 6 – 10 unsaturated isoprenoid units. Phylloquinone is insoluble in water, slightly soluble in ethanol, and highly soluble in oils, fats, and other nonpolar solvents. Vitamin K compounds are highly unstable in basic and acidic media, breaking down under visible and ultraviolet light. However, they are stable at high temperatures, and unlike previous vitamins, they remain stable in the air. Since the 1980s, the primary method for determining vitamin K in food samples has been HPLC with a UV-VIS detector due to the naphthoquinone ring's ability to absorb UV radiation between 240 and 280 nm and 320 and 330 nm [25–27].

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2. Materials and Methods

This study aimed to determine the optimal procedure for extracting samples of baby food for the simultaneous analysis of fat-soluble vitamins (A, D, E, and K) using high-performance liquid chromatography (HPLC). For this purpose, four types of baby food samples were selected based on their composition and consistency: infant milk, chocolate-flavored oatmeal (porridge), and dry and liquid porridge. The analysis will be conducted using seven different methods, each based on four procedures: saponification, enzymatic hydrolysis, solvent extraction, and solid-phase extraction. The efficiency of sample preparation will be assessed based on declared vitamin values on the products and by adding mixed standards of analyzed vitamins to each selected type of food. The obtained results will be used to evaluate the extraction efficiency of vitamins, i.e., the effectiveness of the applied method.

For this study, commercial food samples intended for children were utilized, sourced from the market in the Republic of Croatia. Transitional infant milk, chocolate-flavored cereal (chokolino), dry milk porridge (five grains with plum), and liquid milk porridge (with apple, pear, and wheat grits) were extracted from circulation for analysis.

All samples were prepared in triplicate, and the presented values in the results of this study represent the mean measurement.

Seven different extraction methods were applied to each sample to isolate vitamins A, D, E, and K.

The sample preparation principle for all seven analytical procedures was consistent and involved the extraction of homogenized samples (2.0 ± 0.05 grams) mixed with 10 ml ultra-pure water and 5 ml hydrochloric acid (c = 4 mol dm-3), along with 10 ml of a solution of n-hexane containing 0.025% BHT. After homogenization on a shaker for 30 minutes, sequential centrifugation for 15 min at 4600 revolutions/min followed. Post-centrifugation, 5 ml of the upper hexane layer was pipetted, evaporated to dryness under a nitrogen stream, and the evaporated extract was dissolved in a 500 µl solution consisting of various solvents that differed in each investigated procedure. After dissolution, the sample was ready for the identification and quantification of each vitamin using high-performance liquid chromatography with UV-VIS detection (HPLC/UV-VIS).

In the first three extraction procedures, the solution used for dissolving the evaporated extract was a mixture of acetonitrile and methanol (75:25). In the fourth procedure, after evaporation, a vacuum system was employed for solid-phase extraction with SPE HLB columns. The columns were conditioned with 1 ml of methanol and 1 ml of ultra-pure water, followed by the application of 4 ml of the sample (upper layer). The column was rinsed with 1.5 ml of 5% methanol and dried under vacuum. After drying, analyte elution (vitamins) was performed with 1 ml of isopropanol:acetonitrile (1:1) and then with 1 ml of 20% ethyl acetate in acetonitrile. The collected eluate was evaporated to dryness under nitrogen at 50 °C, dissolved in 500 µl of acetonitrile: methanol (75:25), mixed on a vibrational mixer for half a minute, filtered through a 0.45 µm pore size membrane filter, and further analyzed by HPLC/UV-VIS.

In the fifth procedure, after the initial sample preparation, SPE C18 columns were used. The columns were conditioned with 10 ml of methanol and 5 ml of ultra-pure water, and then 5 ml of homogenized and centrifuged sample (upper layer) was added. After passing the sample through the column, it was rinsed with 5 ml of 10% methanol solution, followed by vacuum drying of the column and elution of analytes with 6 ml of methanol. The collected eluate was evaporated to dryness under nitrogen at 50 °C and then dissolved in 500 µl of acetonitrile: methanol (75:25), mixed on a vibrational mixer for half a minute, and filtered through a 0.45 µm pore size membrane filter into a sample vial. The prepared sample was analyzed by HPLC with UV-VIS detection.

In the sixth procedure, 0.5 g of citric acid monohydrate, 20 ml of ethanol, and 5 ml of 60% potassium hydroxide solution were added to the weighed sample. Homogenization lasted for 30 minutes, followed by heating in a water bath for 30 minutes at 70 °C. After the bath, the vial was cooled to room temperature, and extraction was performed with 10 ml of hexane:ethyl acetate (85:15) solution, followed by another 30-minute homogenization on a shaker. Centrifugation for 5 minutes at 4600 revolutions/min was conducted. After centrifugation, 5 ml of the upper hexane layer was

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pipetted, evaporated to dryness under nitrogen at 50 °C, and the evaporated extract was dissolved in 500 µl of acetonitrile: methanol (75:25). Identification and quantification of analytes were performed using HPLC/UV-VIS.

The seventh procedure began with weighing the homogenized sample, to which 8 ml of ethanol was added, and the content was extracted for 30 minutes on a shaker. Then, 10 ml of n-hexane solution with 0.025% BHT was added, and the sample was further homogenized for 30 minutes. Subsequently, centrifugation for 15 minutes at 4600 revolutions/min was carried out. Following centrifugation, 5 ml of the upper hexane layer was pipetted, evaporated to dryness under nitrogen at 50 °C, and the evaporated extract was dissolved in 500 µl of acetonitrile: methanol (75:25). The prepared sample was analyzed by HPLC with UV-VIS detection.

The samples were analyzed using a high-performance liquid chromatograph with a UV-VIS detector, model Agilent 1200 Series. The column used was Phenomenex C18 with dimensions 250 mm x 4.6 mm; 5 µm, eluted with a gradient mixture of acetonitrile and methanol. The injection volume was 20 µl, the flow rate was set at 1 ml/min, the column temperature was maintained at 45 °C, and the detection wavelength was set at 235, 265, and 325 nm. After HPLC analysis, the obtained chromatograms were analyzed using the ChemStation program.

3. Results

The obtained values of vitamin mass fractions in the analyzed samples, depending on the preparation method, are presented in Table 1. Additionally, the percentage of vitamins obtained by the analytical procedure in relation to the declared vitamin quantity is provided. All samples were analyzed in triplicate, and the results were processed using the Microsoft Excel 2016 software, displaying the mean value of triplicates, standard deviation (SD), and coefficient of variation (CV). Table 2 illustrates the efficiency of extraction for each vitamin using the HPLC technique, while Table 3 presents the results of sample preparation efficiency concerning the type of vitamin.

Table 1. Vitamin Mass Fractions in Analyzed Samples Based on the Preparation Method.

VITAMINS samples		**A (µg/100g)** mean value± SD; (KV) /D.V	**D (µg/100g)** mean value± SD; (KV) /D.V	**E (mg/100g)** mean value± SD; (KV) /D.V	**K (µg/100g)** mean value± SD; (KV) /D.V
PREPARATION 1	**MILK**	352,33 ± 9,07; (2,58) /460 (76,5%)	4,00 ± 0,14; (3,54) /13 (30,76%)	1,95 ± 0,21; (10,88) /12 (16,2%)	17,00 ± 0,62; (3,67) /38 (44,7%)
	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	6,50 ± 0,57; (8,70) /3,3 (196,9%)	ND /no D.V.
	DRY MILK PORRIDGE	218,33 ± 2,08; (0,95) /375 (58,2%)	1,55 ± 0,21; (13,69) /7,1 (21,8)	6,53 ± 0,32; (4,92) /4,8 (136%)	ND /no D.V.
	LIQUID MILK PORRIDGE	92,27 ± 0,55; (0,60) /65 (141,9%)	0,40 ± 0,00; (0,00) /1,1 (36,6%)	ND /no D.V.	ND /no D.V.
VITAMINS Samples		**A (µg/100g)** mean value± SD; (KV) /D.V	**D (µg/100g)** mean value± SD; (KV) /D.V	**E (mg/100g)** mean value± SD; (KV) /D.V	**K (µg/100g)** mean value± SD; (KV) /D.V
PREPARATION	**MILK**	13,75 ± 1,34; (9,77) /460 (2,9%)	0,30 ± 0,00; (0,00) /13 (2,30%)	0,08 ± 0,04; (47,14) /12 (0,66%)	21,00 ± 1,41; (6,73) /38 (55,2%)

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	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	0,80 ± 0,28; (35,36) /3,3	ND /no D.V.
	DRY MILK PORRIDGE	32,40 ± 0,44; (1,35) /375 (8,6%)	ND /7,1 (0 %)	0,80 ± 0,14; (17,68) /4,8 (166,6%)	ND /no D.V.
	LIQUID MILK PORRIDGE	ND /65	ND /1,1	ND /no D.V.	ND /no D.V.
VITAMINS Samples		**A (µg/100g)**	**D (µg/100g)**	**E (mg/100g)**	**K (µg/100g)**
		mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V
PREPARATION 3	**MILK**	454,33 ± 5,13; (1,13) /460 (98,7%)	9,27 ± 0,35; (3,79) /13 (71,3%)	11,60 ± 0,62; (5,38) /12 (96,6%)	36,33 ± 0,58; (1,59) /38 (95%)
	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	3,27 ± 0,21; (6,37) /33 (102,1%)	ND /no D.V.
	DRY MILK PORRIDGE	279,67 ± 3,51; (1,26) /375 (74,5%)	60,15 ± 0,07; (0,12) /7,1 (847,1%)	3,87 ± 0,06; (1,49) /4,8 (80,6%)	ND /no D.V.
	LIQUID MILK PORRIDGE	59,00 ± 1,00; (1,69) /65 (90%)	0,70 ± 0,00 (0,00) /1,1 (63,6%)	ND /no D.V.	ND /no D.V.
VITAMINS Samples		**A (µg/100g)**	**D (µg/100g)**	**E (mg/100g)**	**K (µg/100g)**
		mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V
PREPARATION 4	**MILK**	199,50 ± 2,12; (1,06) /460 (43,3%)	3,30 ± 0,42; (12,86) /13 (25,3%)	1,05 ± 0,07; (6,73) /12 (8,75%)	5,80 ± 0,14; (2,44) /38 (15.2%)
	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	0,54 ± 0,04; (6,61) 3,3 (16,3%)	ND /no D.V.
	DRY MILK PORRIDGE	13,15 ± 0,78; (5,91) /375 (3,5%)	0,85 ± 0,07; (8,32) /7,1 (11,9%)	3,55 ± 0,07; (1,99) /4,8 (73,9%)	ND /no D.V.
	LIQUID MILK PORRIDGE	3,87 ± 0,15; (3,95) /65 (5,9%)	1,20 ± 0,10; (8,33) 1,1 (109%)	ND /no D.V.	ND /no D.V.
VITAMINS Samples		**A (µg/100g)**	**D (µg/100g)**	**E (mg/100g)**	**K (µg/100g)**
		mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V	mean value± SD; (KV) /D.V
PREPARATION 5	**MILK**	523,50 ±30,41; (5,81) /460 (113,8%)	11,50 ± 0,71; (6,15) /13 (88,4%)	11,75 ± 0,49; (4,21) /12 (97,9%)	37,50 ± 2,12; (5,66) /38 (98,6%)
	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	4,45 ± 0,49; (11,12) /3,3 (134,8)	ND /no D.V.

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	DRY MILK PORRIDGE	404,67±16,80; (4,15) /375 (107,7%)	7,65 ± 0,35; (4,62) /7,1 (107,7%)	4,15 ± 0,07; (1,70) 4,8 (86,4%)	ND /no D.V.
	LIQUID MILK PORRIDGE	59,67 ± 2,52; (4,22) /65 (91,8%)	0,83 ± 0,06; (6,93) /1,1 (75,4%)	ND /no D.V.	ND /no D.V.
VITAMINS Samples		**A (µg/100g)** mean value± SD; (KV) /D.V	**D (µg/100g)** mean value± SD; (KV) /D.V	**E (mg/100g)** mean value± SD; (KV) /D.V	**K (µg/100g)** mean value± SD; (KV) /D.V
PREPARATION 6	**MILK**	425,00±36,10; (8,49) /460 (92,3%)	2,70 ± 0,10; (3,70) /13 (21%)	8,73 ± 0,45; (5,16) /12 (72,7%)	2,05 ± 0,07; (3,45) /38 (5,39%)
	CHOCOLATE-FLAVORED CEREAL	ND /no D.V.	ND /no D.V.	3,03 ± 0,21; (6,86) /3,3 (91,8%)	ND /no D.V.
	DRY MILK PORRIDGE	426,33±20,26; (4,75) /375 (113,6%)	2,00 ± 0,14; (7,07) /13 (15,38%)	1,50 ± 0,14; (9,43) /13 (11,53%)	ND /no D.V.
	LIQUID MILK PORRIDGE	76,40 ± 0,98; (1,29) /65 (117,5%)	0,40 ± 0,00; (0,00) 1,1 (36,3%)	ND /no D.V.	ND /no D.V.

Table 2. Extraction Efficiency of the Analytical Method for Determining Fat-Soluble Vitamins Using HPLC Technique.

	A (µg/100g)	**D (µg/100g)**	**E (mg/100g)**	**K (µg/100g)**
MILK	94%	91%	93%	87%
CHOCOLATE-FLAVORED CEREAL	93%	88%	95%	86%
DRY MILK PORRIDGE	90%	87%	91%	89%
LIQUID MILK PORRIDGE	89%	90%	95%	90%

ND- Not detected. D.V.- The declared value of the vitamins.

Table 3. Display of Sample Preparation Efficiency Regarding Vitamin Type.

Vitamin	The order of sample preparation efficiency
A	**PR. 5** – PR. 3 – PR. 6 – PR. 1 – PR. 7 – PR. 4 – PR. 2
D	**PR. 5** – PR. 7 – PR. 4 – PR. 3 – PR. 6 – PR. 1 – PR. 2
E	**PR. 5** – PR. 3 – PR. 6 – PR. 7 – PR. 4 – PR. 1 – PR. 2
K	**PR. 5** – PR. 3 – PR. 2 – PR. 1 – PR. 4 – PR. 6 – PR. 7

PR. - preparation.

Considering the obtained results, the efficiency of sample preparation for all four vitamins was ranked. For the analysis of vitamins A, D, E, and K in the examined baby food, preparation number 5 proved to be the most effective, followed by preparation number 3 for the analysis of A, E, and K vitamins. As for the analysis of vitamin D, preparation number 7 emerged as the second most

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effective. The preparation ranked third in efficiency for both vitamin A and vitamin E is preparation number 6, while for vitamin D, preparation number 4 is third in effectiveness, and for vitamin K, preparation number 2 holds the third position. Preparation number 1, in terms of efficiency, is fourth for both vitamin A and K, while for vitamin D, preparation number 3 occupies the fourth position, and for vitamin E, preparation number 7 is fourth.

4. Discussion

A well-balanced and high-quality diet is crucial for the seamless growth and development of every individual, particularly during the growth and development of children. Fat-soluble vitamins are responsible for enzymatic and metabolic functions in the body. Imbalances in the levels of specific vitamins can lead to health issues, especially in children [28]. Some studies have indicated that children's food rarely contains the declared quantity of vitamin D, and there are instances of hypervitaminosis D in children who consumed children's food with vitamin D levels exceeding the recommended daily dose for their age group [29]. The highest permitted levels of vitamins in products ready for use as placed on the market, or products prepared according to the manufacturer's instructions, are prescribed by the Regulation [30], with permissible deviations from declared values, including those recommended by the European Union Regulation, set at -35% (lower tolerance limit) and +50% (upper tolerance limit) [31]. This research aimed to determine the optimal sample preparation method for milk and baby food for the routine determination of fat-soluble vitamins. The study included seven different extraction procedures for vitamins A, D, E, and K from milk samples and three baby food samples. From the literature, it is evident that various extraction methods are used for extracting vitamins from the analyzed sample group. Solvent extraction proved to be very efficient, although some authors note the need to improve the accuracy and precision of the method in the validation process [32,33]. Solid-phase extraction, enzymatic hydrolysis, saponification, and alkaline hydrolysis also proved to be effective depending on the type of product [34]. Enzymatic hydrolysis catalyzed by lipase was effective in breaking down esters of fatty acids and esters of vitamins A and E. In previous studies, this preparation method partially converted retinyl palmitate and α-tocopheryl acetate into alcoholic forms, while vitamins D and K remained unchanged. Saponification is commonly used to release bound or esterified forms of vitamins A, E, D, and carotenoids. It is important to adjust saponification conditions to achieve optimal extraction and minimal degradation losses [35]. The saponification process is often used in the preparation of samples with complex matrices, such as baby food. Alkaline hydrolysis breaks ester bonds of interfering substances in fatty foods, such as triglycerides, phospholipids, and sterols, and releases vitamins from the lipoprotein complex. Vitamins A and E in food are most found in the form of esters, such as retinyl acetate, retinyl palmitate, and α-tocopheryl acetate, and through saponification, they convert into their alcoholic forms: retinol and α-tocopherol. Vitamin D remains unchanged, while vitamin K is not stable in a basic medium, and the saponification process is not suitable for its analysis [36].

5. Conclusions

In this study, it was experimentally demonstrated that sample preparation based on reverse-phase extraction, specifically solid-phase extraction (SPE), for preparing baby food samples for the analysis of fat-soluble vitamins proved to be the most efficient. Interfering substances were rapidly and easily separated from the analyte using suitable eluting solvents. All other sample preparation methods yielded inferior results and were less efficient. Based on the obtained results, it can be concluded that for the simultaneous extraction of vitamins A, D, E, and K from milk and baby food, it is advisable to use SPE C18 column extraction with acetonitrile and methanol as eluents.

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