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Automated determination of folate catabolites in human biofluids (urine, breast milk and serum) by on-line SPE–HILIC–MS/MS

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ABSTRACT

A rapid, precise and fully-automated method for analysis of folate (vitamin B9) and its catabolites (*viz.* *p*-aminobenzoylglutamate and its acetamide derivative) in biofluids is here presented. The method is based on on-line hyphenation of solid-phase extraction (SPE) by a Prospekt 2 system with hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS). The method was analytically characterized by estimation of repeatability (RSD, $n = 5$, between 0.5 and 4.1%), accuracy (between 96 and 105%), and sensitivity (limits of quantificantion between 0.3 and 8.3 ng/mL (1.1 and 18.8 pmol/mL) or 0.03 and 0.83 ng (0.11 and 1.88 pmol)). The proposed method is suited for routine analysis of folate catabolites as biomarkers to monitor deficiency of vitamin B9.

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1. Introduction

Folic acid (vitamin B9) is a water-soluble vitamin involved in a broad variety of biological processes, as long as it acts as enzymatic cofactor in transference of methyl-group reactions [1]. Folic acid is responsible for the synthesis of DNA bases and chains; therefore, it is essential for the formation of new cells, especially during periods of rapid cell division and growth, such as infancy and pregnancy [2]. Folic acid is also involved in the synthesis of the heme group of haemoglobin, being required for growth and maturation of red blood cells (RBCs) [3]. Accordingly, severe deficiency of folate leads to numerous diseases associated to hindered cell division processes and deficiency of RBCs, such as megaloblastic anemia, bone marrow, or fetal diseases (*spina bifida*, neural tube defects, etc.) [4]. On the other hand, folate takes part in the synthesis of amino acids serine and methionine. In this sense, there is also evidence of the relationship between folate deficiency and accumulation of homocystein, which is the substrate in the folate-mediated synthesis of methionine. This is currently considered an increased risk factor for cardiovascular disease, atherosclerosis and coronary heart disease [5,6].

Folic acid, mainly present as folate under physiologically normal conditions, is excreted in urine as more polar catabolites [7,8]. Catabolic transformation of folate involves reduction to its chemically unstable tetrahydro form, easily transformed to *p*-aminobenzoylglutamate (*p*ABGA). The final product of the folate catabolism is the acetamide derivative of *p*-aminobenzoylglutamate (a-*p*ABGA), the most abundant folate metabolite in urine and other biofluids, as it lacks of metabolic activity.

The metabolic profile of folate catabolites in biological fluids can provide valuable clinical information about the abundance of this essential vitamin in humans. This can be of especial interest in individuals with increased risk of deficiency, such as neonates or pregnant women. In fact, levels of folate catabolites *p*ABGA and a-*p*ABGA have been found considerably increased in serum and urine during pregnancy [1], when the metabolism of this vitamin is enhanced.

Determination of folic acid has been classically performed by microbiological assays [9,10]. However, these methods are not able to discriminate between different folate species and are also affected by the presence of interferences, including growth inhibitors and some antibiotics [11]. Most of the chromatographic methods developed so far are based on reversed-phase liq-

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uid chromatography–tandem mass spectrometry (RP-LC–MS/MS) [12–15]. However, due to their relatively polar nature, folate catabolites are weakly retained in RP chromatographic columns and poor separation is achieved. Hydrophilic interaction liquid chromatography (HILIC) is gaining popularity in metabolomics since biofluids are mainly aqueous and, therefore, likely to contain polar compounds such as carbohydrates, their phosphorylated derivatives, glycolytic intermediates and organic acids [16]. From the best of our knowledge, only one method for determination of folates by HILIC–MS/MS has been reported and applied to human plasma after in-batch sample preparation steps, including standard addition, protein precipitation, filtration, evaporation and reconstitution [17].

Here we present a fully-automated method for the analysis of folate and catabolites, *p*ABGA and a-*p*ABGA, in biofluids. This method is based on the on-line hyphenation of a solid-phase extraction step, automatically carried out by a Prospeckt 2 system, with HILIC–MS/MS chromatographic separation and detection. This hyphenation allows complete automatic performance of the analytical method with a drastic reduction of the analysis time.

2. Materials and methods

2.1. Reagents

LC–MS grade acetonitrile (Panreac, Barcelona, Spain), ammonium formate (Sigma–Aldrich, St. Louis, MO, USA), and deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) were used for preparation of the chromatographic phase.

Pure standards of folic acid (FA), its metabolite N-(*p*-aminobenzoyl)-L-glutamic acid (*p*ABGA) and the internal standard (IS) – aminopterin – were from Sigma–Aldrich. Individual stock solutions were prepared by dissolving 10 mg of standard in 10-mL 0.2 M potassium phosphate buffer, pH 7.3, containing 0.03% ascorbic acid [17]. The acetamide derivative was prepared from the *p*ABGA metabolite with total conversion [7] by adding 140 μL 50% acetic acid (Panreac) in deionized water (v/v) and 20 μL acetic anhydride (Panreac) to 10 mg *p*ABGA. The reaction mixture was vortexed in the dark at room temperature for 1 h. The remaining acetic acid was evaporated under nitrogen at room temperature, and the a-*p*ABGA standard was reconstituted in 10-mL 0.2 M potassium phosphate buffer, pH 7.3, with 0.03% ascorbic acid. Multistandard solutions were daily prepared for optimization of the analytical steps by dilution of the stock standard solutions in deionized water or 80:20 acetonitrile–water (v/v). Standard solutions were stable at −20 °C for at least one month.

2.2. Sample handling

Samples were kindly donated by healthy volunteers. No restrictions about diet, lifestyle, sex or age were taken into account. Venous blood was collected into a BD Vacutainer® Plus SST tube containing spray-coated silica and polymer gel for serum preparation (Becton Dickinson). The tube was not opened to ambient air and placed in ice or kept refrigerated until processing. Blood samples and milk samples were centrifuged at 4000 × *g* for 10 min at 4 °C. The resulting serum was divided into 1-mL aliquots in sterile containers and stored at −20 °C. Ascorbic acid was immediately added (0.03%, w/v) to prevent oxidation of metabolites. For urine samples, the pH was adjusted to 7.3 ± 0.1 with 3 M NaOH or 1 M HCl and ascorbic acid was added (0.03%, w/v) to 1-mL urine aliquots, which were also frozen at −20 °C until analysis without further preparation.

2.3. Instruments

A centrifuge from Selecta (Barcelona, Spain) was used to separate serum from milk and blood samples. A Prospekt 2 system (Spark Holland, Emmen, The Netherlands), equipped with a MIDAS autosampler, an automatic solid-phase extraction unit (automatic cartridge exchanger, ACE) and a high pressure dispenser syringe (HPD), was used for automated solid-phase extraction. Hysphere MM anion exchange (10 mm × 2 mm, 25–35 μm particle size) cartridges from Spark Holland were used for the SPE step. A Hysphere method development tray, also from Spark Holland, was used to optimize the type of sorbent used in the SPE protocol. The Prospekt 2 system was on-line connected to an Agilent (Palo Alto, CA, USA) 1200 Series LC system, which consists of a binary pump, a vacuum degasser and a thermostated column compartment. After chromatographic separation, detection was performed by means of an Agilent 6410 triple quadrupole mass detector (QqQ), furnished with an electrospray ion (ESI) source. Agilent MassHunter Workstation was the software for data acquisition, qualitative and quantitative analysis, while the Sparklink System controller v. 2.1 Software was used to control the Prospekt units.

Chromatographic separation was carried out by hydrophilic interaction liquid chromatography, using a Luna HILIC column (100 × 4.6 mm, 3 μm particle size) from Phenomenex Inc. (Torrance, California, USA), furnished with a HILIC 2 mm × 4 mm (5 μm particle size) Guard Cartridge System.

2.4. Solid-phase extraction

After addition of the internal standard to a final concentration of 20 nmol/mL, biological fluids were placed at the MIDAS tray, being ready for analysis without further preparation. The entire procedure was automatically performed, in a sequence of steps schemed in Fig. 1 and summarized as follows: (A) the sample loop (100 μL) is filled by passing 200 μL sample, the cartridge is clamped into the ACE unit, which is then solvated and equilibrated by means of the HPD unit with 1 mL methanol at 5 mL/min (step 1) and 2 mL loading solution (2 mM potassium phosphate, pH 7.3 ± 0.1) at 5 mL/min, respectively; then, 1-mL loading solution is passed through the cartridge at 0.5 mL/min (step 2); (B) after switching the autosampler valve, the sample is loaded by passing 1-mL loading solution at 0.5 mL/min through the sample loop and then through the SPE cartridge (step 3); the cartridge is subsequently washed with 1-mL of 10% acetonitrile/water at 2 mL/min (step 4); an input/output signal is established between the Prospekt module and the HPLC system (step 5), which is waiting for an external start, (C) the elution starts by switching the ACE valve 1 to the load position; thus, the chromatographic mobile phase passes through the SPE cartridge and the analytes are eluted to the chromatographic column; after 2-min elution (step 6), the ACE valve 1 is switched again; a cleaning step of the cartridge, with 2-mL methanol (step 7) and 2-mL water (step 8) is finally performed. Thus, the SPE cartridge is ready for reuse. Each SPE cartridge was used for three analyses. The whole SPE process is performed at room temperature in 10 min. The SPE and chromatographic steps are synchronised in order to increase the throughput analysis, which is 5 samples/h.

2.5. Chromatographic separation and mass spectrometry detection

Chromatographic separation was performed isocratically in 14 min. The mobile phase was 20 mM ammonium formate in 80:20 (v/v) acetonitrile–water (pH 7.3). The flow rate and column oven temperature were set at 1 mL/min and 35 °C, respectively. Analyses were carried out in multiple reaction monitoring (MRM) positive ionization mode with nitrogen as drying and nebulizing gas. The

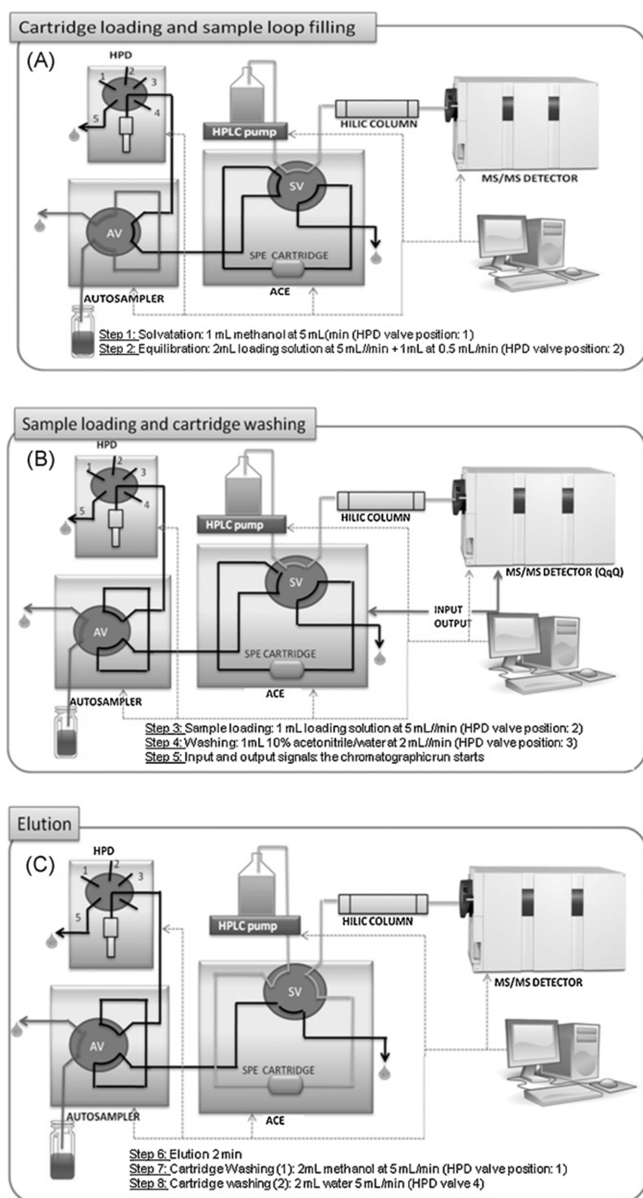

Fig. 1. General scheme of the automatic steps in the Prospeckt 2 system: (A) cartridge loading and solvatation and equilibration, (B) sample loading and cartridge washing (C) elution and washing. HPD: high pressure delivery unit; AV: autosampler valve; ACE: automatic cartridge exchange unit; SV: selection valve.

operating conditions of the ESI–QqQ, were: flow rate and temperature of drying gas 10 mL/min and 300 °C, respectively, nebulizer pressure 45 psi, capillary voltage 4000 V, dwell time 200 ms and delta EMV (potential of the electron multiplier) 700 V. The quantification transition for each compound was: 266.8 → 119.9 *m*/*z* for *p*ABGA, 308.8 → 162.0 *m*/*z* for a-*p*ABGA, 441.2 → 294.2.2 *m*/*z* for FA and 442.2 → 295.1 *m*/*z* for aminopterin (IS). The mass spectra obtained with the individual standard solutions *p*ABGA (A) and a-*p*ABGA is shown in Fig. 2.

3. Results and discussion

3.1. Optimization of chromatographic separation and mass spectrometry detection

Study of the chromatographic separation was performed in positive fullscan detection mode, being the peak separation and shape the parameters considered for optimization. Due to the polar character of the target compounds, HILIC was selected for chromatographic separation as long as it provided an optimum retention and separation. Separation in the HILIC column is performed under high-organic mobile-phase conditions, giving an enhanced ESI performance, which leads to an increased sensitivity and precision. Subsequently, the ionization agent, flow rate and temperature of the column oven were optimized. With this aim, ammonium formate, ammonium acetate and formic acid were tested as ionization reagents, over the concentration range of 5–20 mM. The best results in terms of peak area were obtained with 20 mM ammonium formate in 80:20 acetonitrile–water, which was used further on. The pH of the mobile phase strongly affects the chromatographic separation; thus, pH values between 3 and 9 were investigated, being 7.3 pH the optimum value. Flow rates between 0.5 and 1.5 mL/min and temperatures between 20 and 40 °C were also tested, being 1 mL/min and 40 °C, respectively, the optimum values.

The influence of the operating conditions of the mass spectrometer – namely the temperature of the drying gas, capillary voltage and nebulizer pressure – on the peak area was studied by a multivariate design in order to take into account possible dependence relationships of these variables, being the optimum 250 °C, 3700 V and 45 psi, respectively. Optimization of the best MRM transitions for the analytes and the internal standard was performed with 10 μg/mL multistandard solutions in a sequence of steps that involved the selection of the polarity, precursor and product ions, voltage of the first quadrupole, collision energy and dwell time. The optimization of detection is summarized in Table 1.

3.2. Optimization of solid-phase extraction and on-line elution to the liquid chromatograph

The complexity of biological fluids demands for a suitable sample preparation protocol able to remove the effect of potential interferences prior to chromatographic separation. In this sense, analysis of urine samples is marked by its high-salt content that causes ionization suppression and also negatively affects the instrument performance due to the formation of non-volatile residues. On the other hand, analysis of serum and milk samples is limited by the presence of a large number of proteins that interfere in the chromatographic separation and mass detection if they are not removed [18]. This interference makes the analysis of folate and catabolites especially difficult as long as they are present at very low concentrations. Accordingly, direct analysis of biofluids is rarely performed. Garbis et al. developed a method for determination of folates in human serum based on direct analysis after deproteinization, filtration, vacuum evaporation and reconstitution of the sample [17]. However, the manual performance of these steps enhances the risk of contamination and analyte losses as biological materials are directly handled in all operations. Solid-phase extraction provides clean and concentrated extracts and the possibility of fully-automated on-line implementation.

Optimization of the SPE is summarized in Table 2. Firstly, selection of the SPE sorbent was carried out by using a sample development method tray with the following cartridges: silica-based cyanopropyl phase (CN-SE), silica-based ethyl phase (C2-SE), end-capped silica-based octyl phase (C8 EC-SE), high-density end-capped silica-based octadecyl phase (C18 HD), polydivinyl-benzene phase (resin GP), strongly hydrophobic polystyrene-divinylbenzene phase (resin SH) and mixed-mode phase containing a strong anion exchange functional group (MM anion). This study was performed with both 10 μg/L multistandard solutions and spiked samples in order to check the influence of matrix effects. The poorest retention was obtained with CN-SE, C2-SE, resin SH and resin GP, while MM anion gave the best results in

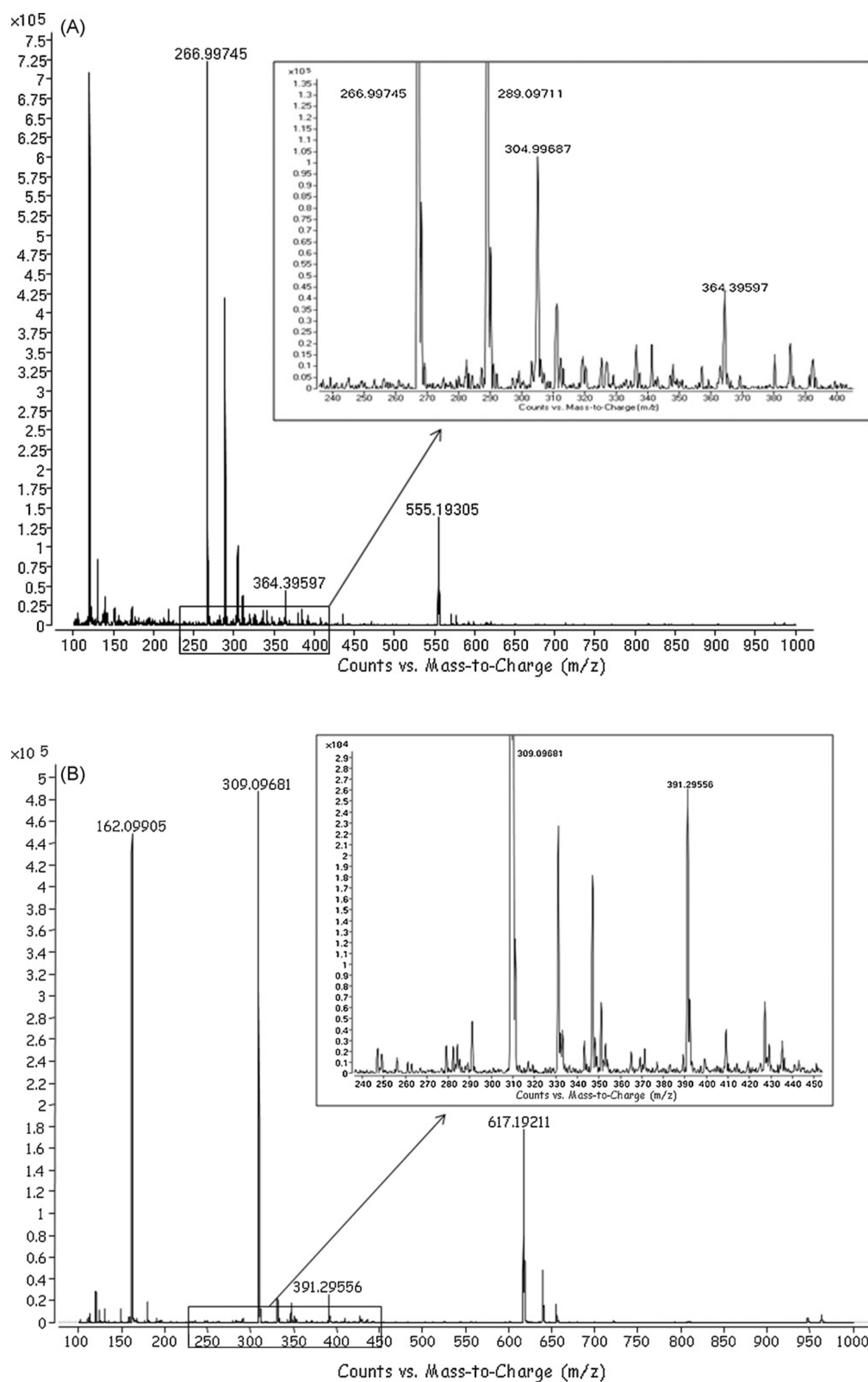

Fig. 2. Mass spectra obtained with individual standards: (A) *p*ABGA and (B) a-*p*ABGA.

Table 1
Optimization of mass spectrometry operation parameters for the multiple reaction monitoring.

Analyte	Molecular weight (g/mol)	Q1 *m/z*	Q3 *m/z*	Retention time (min)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
FA	441.4	442.2	295.1	8.5	140	17	200
*p*ABGA	266.0	266.8	119.9	5.0	120	15	200
a-*p*ABGA	308.0	308.8	162.0	5.2	120	8	200
Aminopterin (IS)	440.4	441.2	294.2	10.5	140	22	200

Table 2
Optimization of the main variables involved in the SPE step.

Variable	Tested range	Optimum condition	Selected condition
SPE sorbent	CN-SE, C2-SE, C8 EC-SE, C18 HD, resin GP, resin SH, MM anion and MM cation	MM anion	MM anion
pH	4–9	Above 6	7.3
Amount of acetonitrile in the sample (%)	0–30	Non-influential	0
Loading flow rate (mL/min)	0.2–2	0.5	0.5
Amount of acetonitrile in the washing solution (%)	0–30	10	10
Washing volume (mL)	0–3	Non-influential	1
Washing flow rate (mL/min)	0.2–2	Non-influential	2
Sample loading flow rate (mL/min)	0.2–2	0.5	0.5
Elution time (min)	0.2–4	2	2

terms of area and peak shape, as the analytes are present as anionic species at weakly basic pH. The MM anion phase is specially suited for extraction of organic acids since it combines a dual interaction based on ionic and polymeric mechanisms responsible of a high retention capacity.

Other relevant parameters optimized were those related with the selection of the sample loading conditions such as pH, concentration of acetonitrile and loading flow rate. For optimization of the sample pH, values within 3 and 9 were tested, for which the appropriate amounts of formic acid, ammonium phosphate buffer (0.2 M, pH 7.3) or NaOH were added. The maximum retention capability was at pH values above 6. Therefore, physiological blood and milk serum pH at 7.3 was selected. In case of urine samples with a pH range from 4 to 8, samples were buffered to pH 7.3 by adjusting with NaOH or formic acid. On the other hand, many protocols for analysis of serum and milk include the addition of acetonitrile or other reagents that favour proteins precipitation. Pre-precipitation of proteins was tested by adding different amounts of acetonitrile to biofluid samples spiked with the target compounds. However, similar results were obtained with and without protein precipitation, which suggests that proteins do not interfere in the extraction process, so this step was not required. Finally, the influence of the flow rate for sample loading was studied by testing flow rates between 0.2 and 2 mL/min, being the optimum value 0.5 mL/min.

Optimization of the washing step involved the selection of the optimum flow rate, volume, and composition of the solution. Flow rate and volume were not influential, so that they were fixed at 2 mL/min and 1 mL. The washing solution was 10% acetonitrile in water, in order to improve the effectiveness of the washing step without analyte losses. Concerning recovery of the target compounds from the SPE cartridge, the elution time was studied within 0.2 and 4 min. The recovery was approached to 100% with 2-min elution times and levelled off for longer times, thus, being minimized to 2 min.

Characterization of SPE protocol was completed by assessment of the breakthrough volume. For this purpose, multistandards at increasing concentrations of the target compounds were analysed by using an experimental setup with two cartridges in an on-line configuration. For this purpose, a second MM anion cartridge was clamped in series by means of an additional selection valve [19]. Thus, the breakthrough volume, expressed as the maximum mass of compounds that can be loaded without giving a quantifiable signal in the elution of the second cartridge, was 10 μg for folic acid, 30 μg for *p*ABGA and a-*p*ABGA and 5 μg for the internal standard. The same study applied to the target biofluids limited the sample loop volume at 100 μL in order to preserve the retention capability.

3.3. Features of the method

Recovery was also evaluated with the double-cartridge configuration. Firstly, the cartridges were conditioned and equilibrated prior to sample loading. If the analytes were not completely

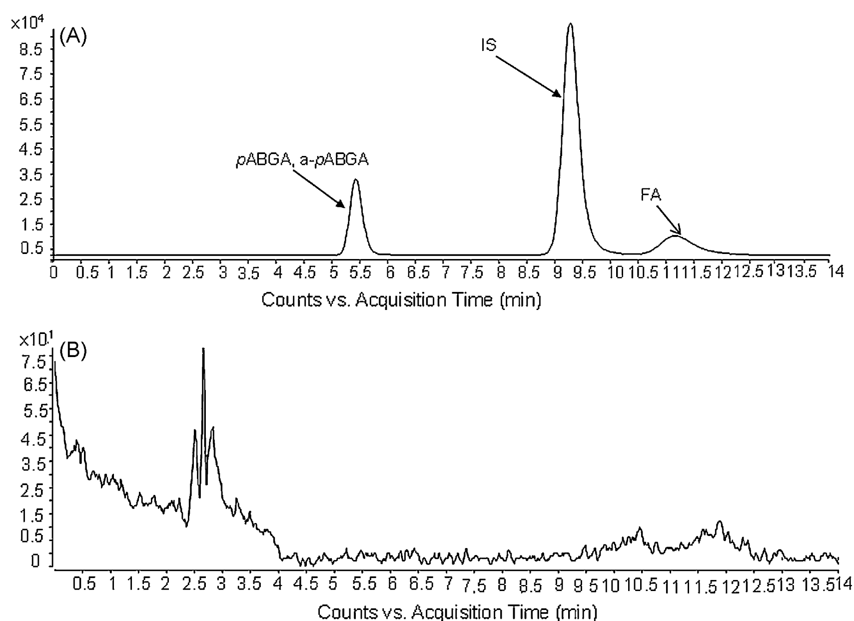

Fig. 3. TIC MRM chromatograms obtained with a 1 μg/mL multistandard solution from a two-cartridge configuration of the Prospeckt 2 system for assessing recovery: (A) elution from the first cartridge; (B) elution from the second cartridge.

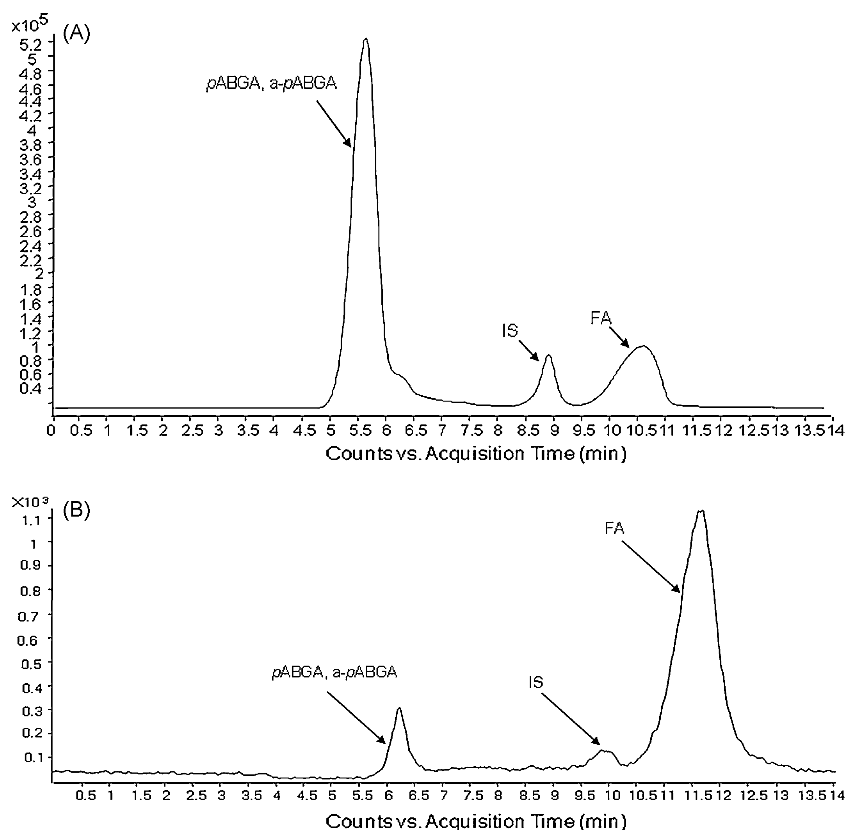

Fig. 4. TIC MRM chromatograms obtained with a multistandard solution at 20 μg/mL of each analyte eluted from the first (A) and the second cartridge (B).

retained in cartridge 1 or saturation is produced, retention in cartridge 2 occurs. Then, the second valve is switched and the mobile phase is loaded through cartridge 1 onto the chromatographic column. Valve 2 is finally switched for elution of the analytes from the second cartridge. Therefore, the maximum recovery is obtained when the MRM signals from the elution of the second cartridge are minimized. Fig. 3 shows the chromatograms obtained with a 1 μg/mL multistandard solution from the first (Fig. 3A) and second (Fig. 3B) cartridge elution. Signals obtained from the second cartridge were not quantifiable; thus, the retention capability was supposed to be 100% for all the target compounds. As Fig. 3A illustrates, a band broadening effect is observed in FA peak signal, which can be ascribed to the mixed interaction of MM anion phase.

On the other hand, Fig. 4 shows the chromatograms obtained with a multistandard solution at concentrations above the breakthrough volume (20 μg/mL of each analyte) from the first and the second cartridge. In this case, retention in the first cartridge was not complete as detected in the chromatogram generated by analysis of the elution fraction from the second cartridge. In order to assess the recovery, urine samples spiked at two concentration levels – 50 and 250 ng/mL – and a blank were analysed. Accuracy was investigated by comparing theoretical and experimentally measured analyte concentrations obtained from spiked samples at the two levels. The results obtained for both parameters are summarized in Table 3.

Calibration plots were run by using the standard peak–internal standard peak ratio as a function of the standard concentration. Calibration was performed with multistandard solutions at ten concentration levels – between 0.1 and 10,000 ng/mL – which were analysed in triplicate. The regression coefficients and the dynamic range are shown in Table 3. The lower limits of detection (LLOD), expressed as the mass of analyte which gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal) ranged between 0.3 and 5.5 pmol/mL (0.03 and 0.55 pmol on column). The lower limits of quantification (LLOQ), expressed as the mass of analyte which gives a signal that is 10σ above the mean blank signal, ranged between 1.1 and 18.8 pmol/mL (0.11 and 1.88 pmol on column).

The precision, expressed as repeatability, was estimated by analysis of five aliquots of spiked samples at 50 ng/mL (FA: 0.12 nmol/mL, *p*ABGA: 0.19 nmol/mL and a-*p*ABGA: 0.16 nmol/mL) and 250 ng/mL (FA: 0.60 nmol/mL, *p*ABGA: 0.94 nmol/mL and a-*p*ABGA: 0.81 nmol/mL) in a single working session. As can be seen

Table 3
Features of the proposed method.

Analyte	LLOD (pmol/mL)	LLOQ (pmol/mL)	Linear regression (r^2)	Dynamic range	Precision at low level[a] (RSD%, n = 5)	Precision at high level[b] (RSD%, n = 5)	Accuracy at low level[c] (%)	Accuracy at high level[c] (%)
*p*ABGA	0.3	1.1	0.9999	10^3	2.5	1.7	96	100
a-*p*ABGA	0.6	1.9	0.9996	10^3	3.2	3.8	102	100
FA	5.5	18.8	0.9998	10^4	4.1	2.8	101	105

[a] Low level: urine pool spiked at 50 ng/mL (FA: 0.12 nmol/mL, *p*ABGA: 0.19 nmol/mL and a-*p*ABGA: 0.16 nmol/mL).
[b] High level: urine pool spiked at 250 ng/mL (FA: 0.60 nmol/mL, *p*ABGA: 0.94 nmol/mL and a-*p*ABGA: 0.81 nmol/mL).
[c] Accuracy (%): 100 × (measured concentration – blank concentration)/spiked concentration.

Table 4
Mean values and standard deviation of the analysis of five urine, blood and milk serum samples.

Analyte	Urine (pmol/mL)	Blood serum (pmol/mL)	Breast milk serum (pmol/mL)
a-*p*ABGA	87.7 ± 1.4	165.4 ± 1.3	145.6 ± 2.3
*p*ABGA	23.9 ± 0.7	19.6 ± 0.4	64.5 ± 1.6
FA	20.5 ± 1.5	98.7 ± 1.8	41.7 ± 1.4

in Table 3, the results obtained, expressed as relative standard deviation (RSD), were from 2.5 to 4.1% and 1.5 to 3.8% for the low and high level, respectively.

3.4. Application of the method to human biofluids

The proposed method was tested by application to human biofluids in order to check its suitability for clinical analysis. Three biofluids such as serum, urine and human milk were selected for analysis following the developed method. Five independent replicates were made for each biofluid ($n = 5$). Table 4 shows the mean value and the standard deviation obtained for the target compounds in each sample, where all the folates were present at quantifiable levels. Most studies dealing with folate deficiency have been carried out by radioimmunoassay kits for serum analysis. The application of these kits has set a deficiency cut-off for folate

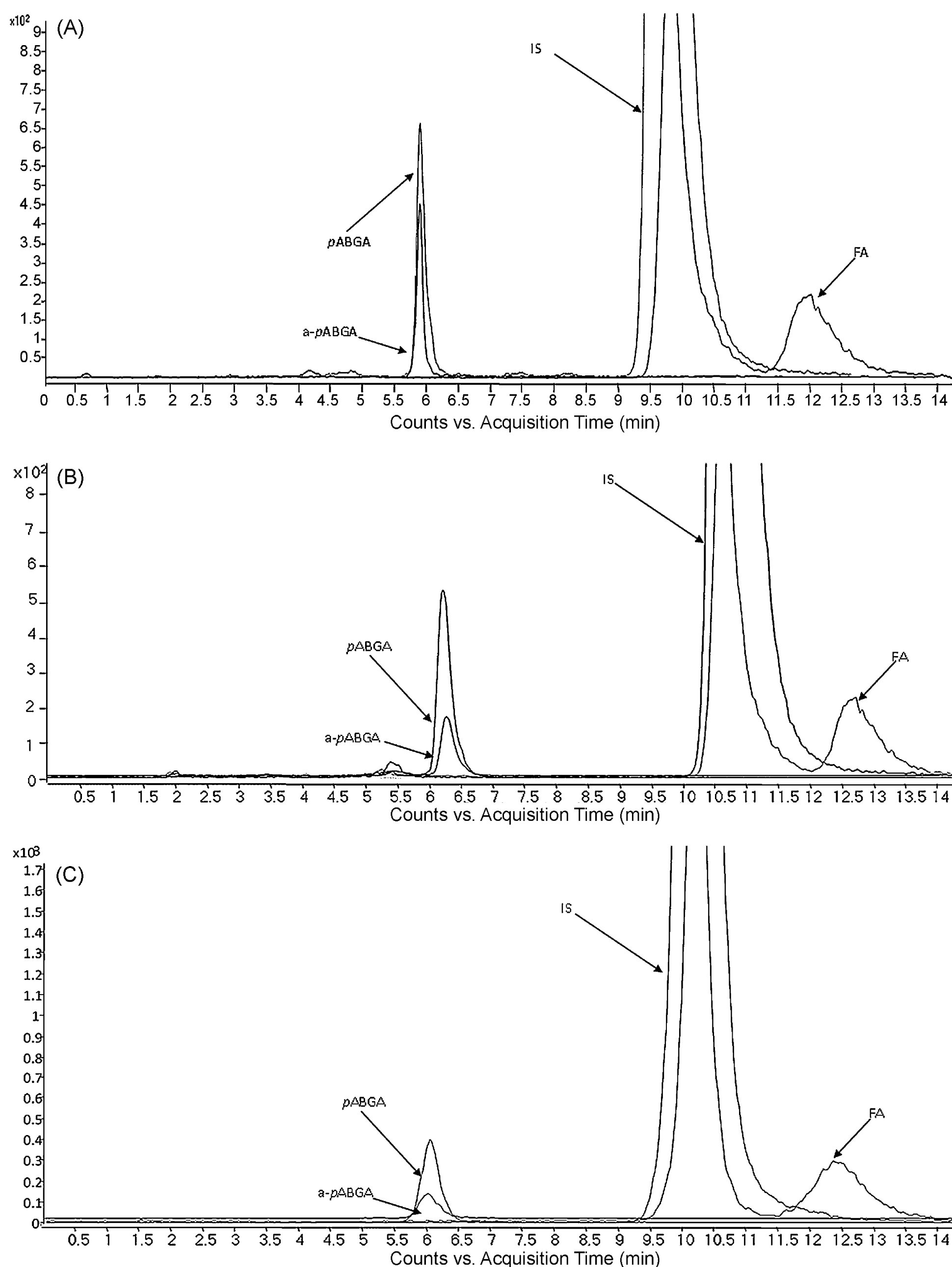

Fig. 5. MRM chromatograms provided by blood serum (A), milk serum (B) and urine (C) samples analysed by the proposed method.

concentrations below 11 pmol/mL [20]. As indicated in Section 1, these assays are not specific and, therefore, respond to associated metabolites. Therefore, taking into account LLODs and LLOQs reported here for folate and metabolites, the proposed method is specially suited for serum analysis. As Fig. 5A shows, folates were successfully analysed in serum without chemical effect of interferences caused by high concentration of proteins. Similarly, the proposed method can be applied to human breast milk as Fig. 5B proves. In both cases, a-*p*ABGA was the metabolite found at higher concentration.

Folic acid is predominantly excreted in urine as more polar catabolite. Therefore, the analysis of folates in this biofluid could be interesting. Urine is a representative biological matrix with a high-salt content and, for this reason, sample preparation is a critical task. As Fig. 5C shows, cleaning and desalting were efficiently performed by the SPE-based approach proposed here. Mass spectrometry analysis was not affected by ionization suppression.

4. Conclusions

A method for analysis of folic acid and its main catabolites, *p*-aminobenzoylglutamate and the acetamide derivative in biofluids has been developed. The method, based on the on-line hyphenation of a solid-phase extraction step with HILIC–MS/MS chromatographic separation and detection, is fully automated, and no in-batch operations, such as protein precipitation or filtration, were required.

The entire analytical process has been carefully optimized and characterized. Subsequently, it has been applied to the analysis of the target compounds in urine, breast milk and serum samples. Although the target analytes were present in the three biofluids, the most abundant folate catabolite in all samples is a-*p*ABGA. On the other hand, the highest concentration of folic acid is found in serum. In fact, it is well established that serum is likely to reflect the metabolic state of an organism as a consequence of both catabolic and anabolic processes occurring in the whole organism, while excreted biofluids, such as urine, provides readout of catabolic processes, in which polar metabolites without metabolic activity are more readily discarded from the body.

A metabolic profile of these compounds in the target samples offers valuable information about the abundance of this essential vitamin in the human body. This can be of especial interest in groups of individuals with increased risk of deficiency (such as neonates or pregnant women). Comparison between the levels of these related catabolites in the three types of biofluids gives a realistic readout of the occurrence and fate of this water-soluble vitamin in humans.

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