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Pressurized Liquid Extraction and HPLC Quantification of Folic Acid in Fortified Wheat Flours

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ABSTRACT: A pressurized liquid extraction (PLE) method using phosphate buffer as solvent was applied for folic acid (FA) extraction from fortified wheat flours and was compared to a standard solid−liquid extraction (SLE) method. Extracted FA was quantified by reverse phase high-performance liquid chromatography (RP-HPLC) hyphenated with a phenyl column and an absorption photometric detector (λ = 280 nm). Detection and quantification limits were 0.12 and 0.4 ng, respectively, corresponding to 0.06 and 0.2 μg g^{-1} of analyzed wheat flour. Equivalent FA contents were found by both extraction methods, but a single PLE allowed a total recovery of FA content, whereas at least three successive SLEs were needed to achieve a total recovery of FA. The obtained results indicated that PLE is a rapid and efficient technique for FA extraction from fortified wheat flour.

KEYWORDS: *folic acid, wheat flour, pressurized liquid extraction, HPLC*

INTRODUCTION

Folate is a B-vitamin, recognized for its role in providing one-carbon units for nucleotide synthesis and repair and for all cell replications including normal fetus development.[1] "Folic acid" (FA), the term often used interchangeably with "folate", represents only a small percentage of the naturally occurring folates, but its stability and availability account for its widespread use in fortification of foods and the preparation of vitamin supplements.[2]

Nowadays, due to an increasing number of fortified foods on the market and because of its importance in the health of the general population, reliable and quick methods are needed to extract and quantify FA in fortified cereals.[3,4] Usually, folate extraction is timing-consuming and involves a series of enzymatic steps to release the native folates bound to the food matrices and to deconjugate polyglutamyl folates to mono or other simpler measurable forms. In fortified cereals, no enzymatic steps are necessary as FA is the major form of folate as cereals generally contain low levels of native folate.[5] Therefore, a simple solid−liquid extraction (SLE) may be sufficient to recover quantitatively the fortified FA.

Among the new alternative extraction techniques that are available is pressurized liquid extraction (PLE), which has received particular attention recently and gained wide acceptance for the extraction of organic compounds.[6] This technique has been already used to extract compounds from various biological materials, such as mycotoxins,[7] saponins,[8] oils,[9,10] polyphenols,[11] lipids,[6,12] veterinary drugs,[13] pesticides,[14] and antivirals.[15] The PLE technique uses small amounts of conventional solvents at elevated temperatures and pressures to achieve quantitative extraction from solid and semisolid samples in a short time.[16−18]

The PLE technique was used for the first time to extract FA from different fortified wheat flours in this paper. The FA quantification was achieved by reverse phase high-performance liquid chromatography (RP-HPLC). The FA contents were determined and compared to these obtained with the traditional SLE.

MATERIALS AND METHODS

Materials. *Chemicals.* Acetonitrile (ACN) (Sigma-Aldrich, Steinheim, Germany) was of HPLC grade. All other chemicals were of analytical grade. Acetic acid was purchased from Riedel-de Haen/Fluka (Seelze, Germany), sodium hydroxide (NaOH) from SDS (Peypin, France), and dipotassium hydrogen phosphate trihydrate from Carlo Erba (Val de Reuil, France). FA and theophylline (TP) were obtained from Sigma-Aldrich. Water was purified using a Synergy Milli-Q System (Millipore, Molsheim, France). Sand (Fontainebleau) was provided by VWR (Leuven, Belgium).

Food Samples. Six commercial Brazilian wheat flours fortified with FA were purchased in different local supermarkets in São Paulo city (Brazil) from July to December 2010.

Nonfortified wheat flours were purchased in local supermarkets in Strasbourg (France) in March 2011 and served as wheat flour blank samples. They were fortified in-house with FA to final concentrations of 0.75, 1.5, and 3.0 μg g^{-1}. Blending was carried out with a Turbula T 2 F shaker (Wab, Basel, Switzerland) at a speed of 72 rpm and a fill volume of approximately 25%. First, 300 mg of FA was blended with 200 g of nonfortified wheat flour for 17 min, resulting in a final concentration of 1500 μg g^{-1} (flour A). Second, 10 g of flour A was blended with 90 g of nonfortified wheat flour for 5 min, resulting in a

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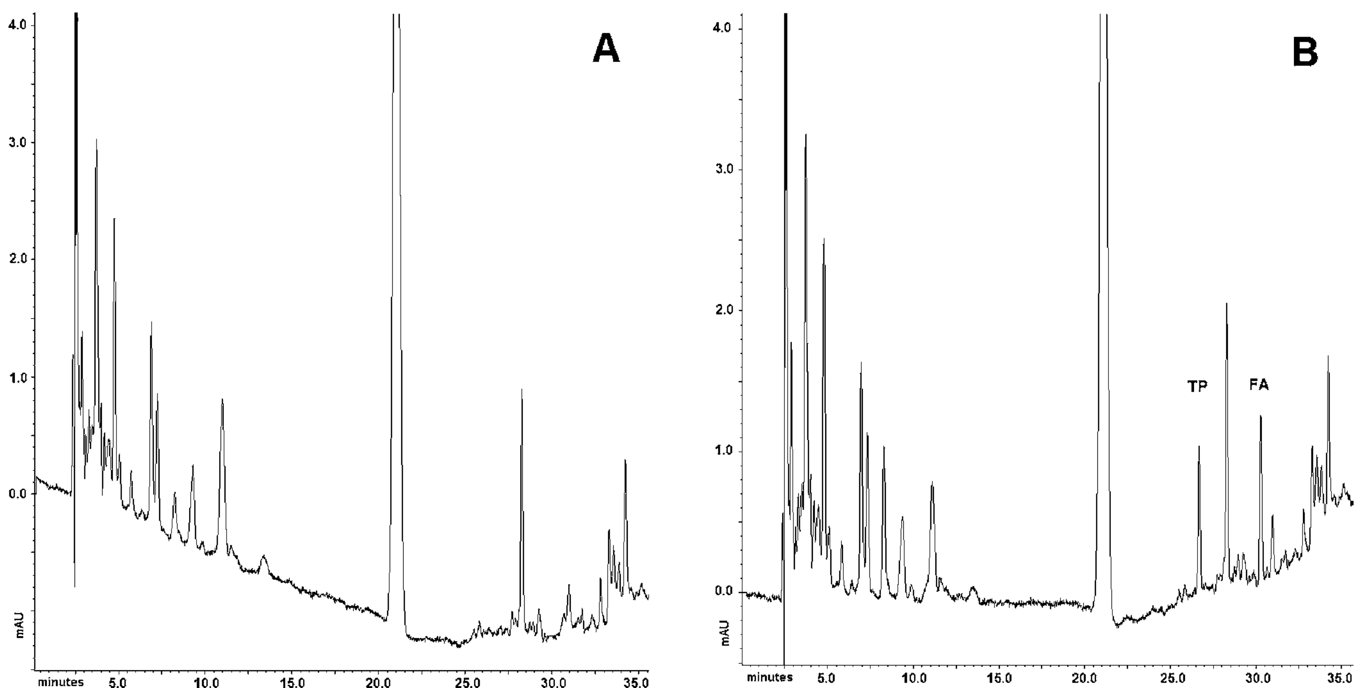

Figure 1. HPLC chromatograms of (A) nonfortified wheat flour extract without internal standard and (B) FA fortified wheat flour extract with TP as internal standard.

final concentration of 150 μg g^{-1} (flour B). Finally, 1 g of flour B was blended with 99 g of nonfortified wheat flour for 5 min, resulting in a final concentration of 1.5 μg g^{-1}. Similarly, final concentrations of 0.75 and 3.0 μg g^{-1} were reached by blending 0.5 and 2.0 g of flour B with 99.5 and 98.0 g of nonfortified wheat flour for 5 min, respectively. All preparations were carried out in triplicate.

Sample Preparation. *Solid–Liquid Extraction.* The analytical protocol was adapted from Alaburda et al.[19] Briefly, 3.0 g of wheat flour samples was mixed with 30 mL of 0.1 M phosphate buffer, pH 9.0. The mixture was shaken at room temperature for 30 min in a rotational shaker and then centrifuged at 3500 rpm for 15 min. The supernatant was removed, and the pellet was re-extracted three times (total of four extractions) following the same procedure. Each supernatant was filtered through a 0.22 μm hydrophilic PVDF membrane (Millipore, Carrigtwohill, Ireland) before chromatographic analysis. Each individual wheat flour sample was analyzed in triplicate.

Pressurized Liquid Extraction. PLE was performed on a Dionex PLE 350 (Dionex, Sunnyvale, CA, USA) system. An amount of 3.0 g of wheat flour was homogenously mixed with 41 g of sand so as to fill the 22 mL stainless steel extraction cell (L × ⌀: 52 mm × 28 mm). A volume of 50 μL of a 100 μg mL^{-1} TP solution was added to each filled extraction cell. A cellulose filter was placed in the cell outlet. The phosphate buffer previously used in SLE was employed for PLE. Different extraction temperatures (40−80 °C) were investigated. The extraction pressure was 10 MPa. Each automated extraction comprised three cycles with 3 min of static time. Following each cycle, the cell was rinsed with fresh extraction solvent (20% of the extraction cell volume). Finally, after the third static time, the cell was purged with a flow of nitrogen (10 MPa during 60 s). The total extraction procedure took 15 min. The same sample was re-extracted two times (without the addition of TP solution). Each extract (around 30 mL) was recovered in different vials and filtered through a 0.22 μm hydrophilic PVDF membrane (Millipore) before chromatographic analysis. Each individual wheat flour sample was analyzed in triplicate.

High-Performance Liquid Chromatography. Chromatography work was performed using a Beckmann Coulter System Gold HPLC system (Palo Alto, CA, USA) (programmable solvent module 126 pump, autosampler 508, and DAD module 168 detector). Chromatographic separation was carried out on an Agilent Zorbax Eclipse XDB-Phenyl analytical column (250 × 4.6 mm; 5 μm particle size) at 20 ± 2 °C. A gradient elution with acetonitrile and aqueous acetic acid (pH 2.8) was used. The flow rate was 1.0 mL min^{-1}. The gradient started at 2% ACN for 15 min. The ACN proportion rose linearly to 25% within 20 min and was maintained for 8 min. After 1 min, the ACN proportion came back to 2% and the column was restabilized for 12 min before the next injection. An injection volume of 20 μL was used. Detection was performed at 280 nm. To control the analytical system, water and FA solution in phosphate buffer at 0.15 μg mL^{-1} were injected throughout the sample analysis every 10 injections. FA identity was controlled by its UV spectra (DAD detection).

Quantification of FA in Fortified Wheat Flours. Quantification of FA was performed either by external calibration with FA standard for SLE method or by internal calibration with FA and TP standards for PLE method. Linear calibration curves were obtained over the ranges evaluated for both external and internal standards. For both calibrations, a solution of FA at 1000 μg mL^{-1} was first prepared in 0.1 M phosphate buffer, pH 9.0, and then diluted to obtain a solution at a final concentration of 2 μg mL^{-1}. The latter was used to prepare FA calibration solutions at six different concentrations ranging from 0.02 to 0.3 μg mL^{-1}. For the internal calibration, the internal standard TP was added in each FA calibration solution to obtain a final concentration of 0.16 μg mL^{-1}. Linear calibration curves were obtained over the ranges evaluated for both external and internal standards. All FA standard solutions were prepared under subdued light and stored in amber vials to protect FA from light oxidative degradation.

The limit of detection (LOD) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 10.

Wheat flours were analyzed in triplicate. FA contents were expressed in μg g^{-1} of wheat flour. All results are given as the mean ± standard deviation (SD). The Student *t* test was used to determine significant differences between FA contents with a confidence level of 95%.

■ RESULTS AND DISCUSSION

Chromatographic Analysis. Unlike other studies using a C_{18} column,[19−22] the chosen phenyl column showed superior

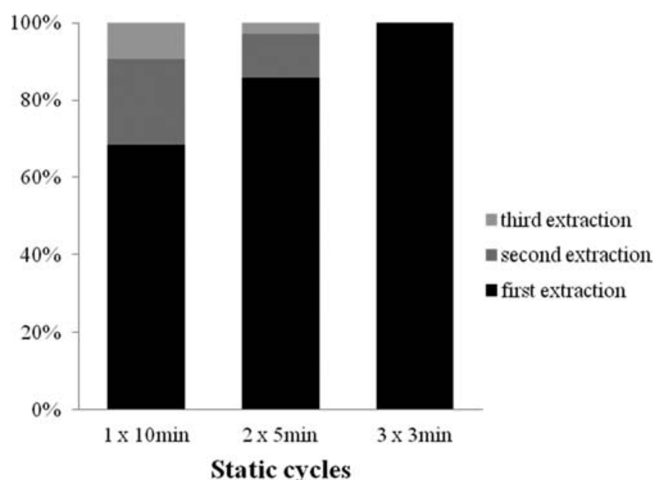

Figure 2. Recovery percentage of FA after PLE optimization.

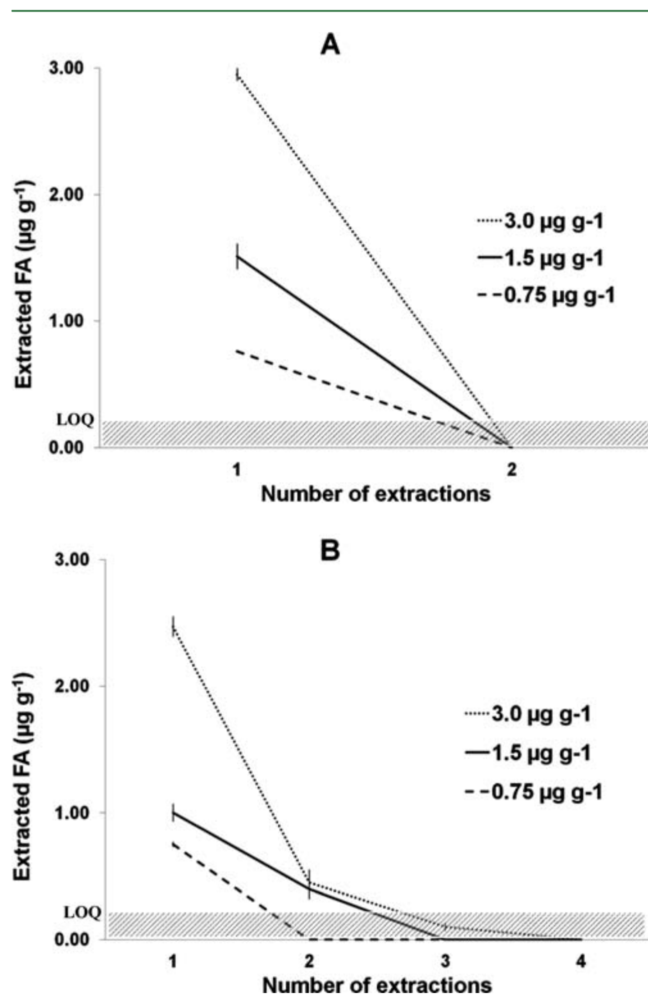

Figure 3. Amounts of FA recovered in successive extractions of in-house-fortified wheat flours: (A) PLE method; (B) SLE method.

selectivity and sensitivity toward FA. Our HPLC method showed LOD and LOQ values of 0.12 and 0.4 ng, respectively, corresponding to 0.06 and 0.2 $\mu g\ g^{-1}$ of analyzed wheat flour. A tested Hypersil Gold aQ C_{18} column (250 × 4.6 mm; 5 μm) showed LOD and LOQ values 2−3 times higher. Besides, whereas FA showed not enough affinity to a Macherey-Nagel CN column (125 × 3.0 mm; 3 μm), FA was tightly retained on a Nucleosil 100-3 NH_2 column (125 × 4.6 mm; 3 μm).

Table 1. Recovery Values of FA Extracted by PLE and SLE from Reference Fortified Wheat Flour

theor FA content ($\mu g\ g^{-1}$)	extraction method	FA content ($\mu g\ g^{-1}$)	recovery (%)
0.75	PLE	0.76a ± 0.01	101.0 ± 1.7
	SLE[a]	0.73a ± 0.01	97.3 ± 2.0
1.5	PLE	1.51b ± 0.10	101.0 ± 6.4
	SLE[a]	1.39b ± 0.06	92.7 ± 3.9
3.0	PLE	2.95c ± 0.05	98.3 ± 1.7
	SLE[a]	2.93c ± 0.14	97.7 ± 5.5

[a]Results of three successive SLEs. Values are expressed as the mean ± standard deviation (n = 3). Means followed by the same letter indicate no significant difference ($p < 0.05$).

Chromatographic profiles of wheat flour blank sample extract (nonfortified wheat flour) and fortified wheat flour extract with internal standard TP are shown in Figure 1. Retention times of FA and TP are 30.4 and 26.6 min, respectively. No peaks were observed at retention times of FA and TP in the chromatogram of blank wheat flour sample (Figure 1A). Consequently, the proposed HPLC method is specific. As pointed out by other authors,[5,22] nonfortified wheat flours does not contain natural FA or its concentration remains below the detection limit of the analytical method. Our results confirm their statement.

An external calibration with FA standards showed a linear calibration curve ($y = 73967x + 97.4$, $r^2 = 0.999$, where y represents FA peak area and x, FA concentration). As well, an internal calibration with FA and TP standards showed r^2 = 0.999 and $y = 1.2573x + 0.0236$. where y represents the peak area ratio FA to TP and x, the concentration ratio of FA to TP.

Extraction Optimization. *Pressurized Liquid Extraction.* Because recovered extraction volume by PLE was variable (around 30 mL), it was necessary to use an internal standard to quantify FA content properly. TP was chosen as an internal standard because of its similar chemical structure and maximum absorption wavelength compared to FA. Pallaroni et al.[7] extracted the mycotoxin zearalenone by PLE from wheat and corn samples and also used an internal standard (zearalanone) due to the variable final volume of PLE extract. Zearalanone was added to the extraction solution, and the volume was filled to 40 mL. Such a procedure was not used in our work because of the important dilution effect, which compromised folic acid LOD and LOQ.

For an efficient PLE, parameters such as solvent, temperature, extraction time, and number of cycles should be studied and optimized. A phosphate buffer was chosen as extraction solvent because FA presented a high solubility and stability in this condition. Moreover, comparing PLE and SLE results became more precise by using the same phosphate buffer previously used in SLEs.

In our study, temperature increase did not yield better FA extraction yields. The best extraction of FA was obtained at 40 °C (minimal temperature for PLE equipment) for 15 min of extraction. A final extracted volume of around 30 mL was recovered. Temperatures >40 °C triggered the aggregation of wheat flour particles and the formation of a compact mixture, which led to a cloudy extract. As a result, the higher the temperature is, the more difficult filtering PLE extracts for

Table 2. Contents of FA by PLE and SLE for Six Different Commercial Fortified Wheat Flours Determined by HPLC

extraction method	contents of FA in commercial wheat flours (μg g^{-1})					
	I	II	III	IV	V	VI
PLE	3.23a ± 0.10	3.05b ± 0.02	2.18c ± 0.13	1.94d ± 0.15	1.78e ± 0.22	1.66f ± 0.04
SLE[a]	3.24a ± 0.36	3.25b ± 0.16	2.32c ± 0.14	1.88d ± 0.13	1.84e ± 0.20	1.60f ± 0.10

[a]Results of three successive SLEs. Values are expressed as the mean ± standard deviation ($n = 3$). Means followed by the same letter within a column indicate no significant difference ($p < 0.05$).

HPLC analysis is. These results can be explained by the fact that wheat flour contains very small particles that can adhere tightly to each other under elevated pressure and temperature and alter extraction efficiency.[23] That is why sand was used as a dispersing agent to assist in the extraction process and ensure a porous mixture that allows the solvent to flow easily through the sample. Pallaroni et al.[7] also emphasized the need for a dispersing agent (diatomaceous earth) to resolve this problem and obtained dirty extracts with increasing temperatures.

The number of extraction cycles was then investigated to achieve a total FA extraction. Neither one cycle of 10 min static time nor two cycles of 5 min static time yielded a complete FA extraction in a single extraction procedure (Figure 2). A total FA content recovery was achieved by using one extraction procedure comprising three cycles of 3 min static time (Figure 2). The latter led to a division of the original flush volume into three cycles, introducing fresh solvent during the extraction process, which helped to maintain favorable extraction equilibrium. As previously highlighted, using the PLE method, FA was totally extracted from fortified wheat flour samples after a single three cycles of extraction. Moreover, the extraction procedure took only 15 min and only 30 mL of solvent was needed to accomplish the extraction.

As shown in Figure 3A, all FA was extracted by the first extraction from fortified wheat flours regardless of their fortification level (0.75, 1.5, or 3.0 μg g^{-1}). The obtained quantity of FA below the LOQ in the second extraction confirmed these results. PLE provided a simple, automatic, fast, and effective technique to extract FA from fortified wheat flours. One extraction (15 min) was sufficient to recover all FA in fortified wheat flour. Moreover, PLE is economical and environmentally friendly, taking into account the reduced solvent consumption and reusable materials.

Solid−Liquid Extraction. SLE needed one, two, even three subsequent extractions with 30 mL of solvent each to reach total FA exhaustion from the wheat flour matrix, depending on the FA fortification level (Figure 3B). Although one extraction recovered almost all FA from the wheat flour fortified with 0.75 μg g^{-1} of FA, three extractions were needed to recover all FA from the wheat flour fortified with 3.0 μg g^{-1}. Gregory[2] also reported that a double-extraction procedure (i.e., residue from the first extraction resuspended and centrifuged) yields more folate than a routine single-extraction procedure. Moreover, the SLE method represents a time-consuming analysis, whereas each solid−liquid extract (first, second, and third extractions of each sample) should be chromatographically analyzed individually to quantify the total FA amount. Indeed, pooling the three extracts together causes a 3 times dilution effect, which compromised FA LOD and LOQ. Besides, a concentration step (for example, evaporation) would delay even more analysis because a nonvolatile high-saline phosphate buffer was employed.

Moreover, contrarily to PLE, the use of an internal standard was impossible for the traditional SLE method because successive extractions were needed to recover all FA, and due to its high solubility in phosphate buffer, all TP added was extracted in the first extraction. As a result, an external standard calibration was applied for the SLE method.

FA Extraction Recoveries from In-House-Fortified Wheat Flour. FA extraction recoveries were evaluated for both PLE and SLE methods with in-house fortified wheat flours at three FA concentrations, 0.75, 1.5, and 3.0 μg g^{-1}. The results shown in Table 1 represent the recovery values of a single extraction for the PLE method, but the sum of three extractions for the SLE method. It was shown that the recovery values ranged from 92.7 to 101.0%. There was no significant difference ($p < 0.05$) to each level of fortification between the different extraction methods (PLE and SLE), indicating that both extraction methods are efficient in extracting FA from wheat flours. Moreover, both extraction methods showed low standard deviation (SD) values as well as low coefficients of variation (ranging from 1.7 to 6.4%).

Determination of FA Contents of Commercial Wheat Flours. Both PLE and SLE methods were applied to commercially available fortified wheat flour products. The amounts of FA (μg g^{-1} of wheat flour) recovered from wheat flour using both PLE and SLE were very consistent (Table 2) and showed a high correlation ($r^2 = 0.9835$) ($p < 0.05$). Results from both extractions methods showed no significant difference ($p < 0.05$) in FA content. With regard to data obtained with the PLE method, FA contents ranged from 1.66 to 3.23 μg g^{-1} in the commercial fortified wheat flours. In all analyzed fortified wheat flour samples, the FA label claim was exceeded. Two commercial fortified wheat flours (I, II) presented an overfortification, which exceeded 2 times the mandatory fortification level (1.50 μg g^{-1}) established by the Brazilian National Health Surveillance Agency (ANVISA)[24] (Table 2). Osseyi et al.[20] and Rychlik[25] also highlighted an over-fortification in fortified cereal products and suggested that this fact could be an assay of the food industry to compensate a possible FA loss during wheat flour fortification and/or FA loss during food processing to guarantee the final concentration established. Another Brazilian legislation[26] establishes the fortification of foodstuffs with vitamins and minerals until 100% of dietary reference intake, which means 2.40 μg g^{-1}. Some fortified wheat flours exceed this threshold. Even so, it is already known and documented that worldwide FA recommended levels for fortification reasons are extremely inferior to the tolerable upper intake level of 1000 μg day^{-1} for adults.[27]

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Notes

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■ ABBREVIATIONS USED

FA, folic acid; PLE, pressurized liquid extraction; SLE, solid−liquid extraction; TP, theophylline; RP-HPLC, reversed phase high-performance liquid chromatography; HPLC, high-performance liquid chromatography; ACN, acetonitrile.

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