Analysis of Theobromine and Related Compounds by Reversed Phase High-Performance Liquid Chromatography with Ultraviolet Detection: An Update (1992–2011)

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Summary

Theobromine and its related compounds, such as caffeine and theophylline, are secondary metabolites that belong to the alkaloids and have economic and cultural importance. These alkaloids have demonstrated stimulatory effects on the central nervous, gastrointestinal, cardiovascular, renal and respiratory systems, resulting in 'energy arousal', increased motivation to work, increased alertness and increased cognitive function. Several analytical methods have been used to analyse these compounds, but reversed phase high-performance liquid chromatography (RP-HPLC) is the most commonly applied because of its efficiency, sensitivity, specificity and speed. This review describes the analyses of theobromine-related compounds by RP-HPLC with ultraviolet detection (UV) in four sources: food, beverages, biological fluids and plants. Many RP-HPLC methods have been developed and optimized for the detection and quantification of these natural compounds. Elution under isocratic conditions is the most frequent method, with a water, methanol and acetonitrile mixture modified with acetic, phosphoric or formic acid as the mobile phase. For xanthine analysis, the use of reversed phase high-performance liquid chromatography with an ultraviolet/diode array detector (UV/DAD) is particularly suitable as derivation is not required; it allows the analysis of absorbance at all wavelengths, it is simple and rapid.

Key words: theobromine, RP-HPLC, beverages, biological fluids, plants

Introduction

Purine alkaloids are secondary metabolites derived from purine nucleotides (*1*). These compounds have been found in nearly 100 different plants of several families, in some cases without a phylogenetic report (*2–4*). The most important representatives of this class are the derivatives of xanthines, such as theobromine (a), caffeine (b) and theophylline (c) (*4,5*), shown in Fig. 1.

The distribution of theobromine and its related compounds is restricted mainly to plant families cultivated in tropical and subtropical regions. Approximately 60 species are cultivated to allow the large-scale production of foods that are consumed daily. A variety of foods and beverages included in this group are obtained from coffee beans (*Coffea* sp.), black tea (*Camellia sinensis* L.), mate leaves (*Ilex paraguariensis*), guarana (*Paullinia cupana*) and cocoa seeds (*Theobroma cacao*) (*6–10*). These products, which are of major economic and cultural importance, contain a variety of interesting methylxanthines.

In recent years, derivatives of xanthines have received increasing attention as components of the so-called

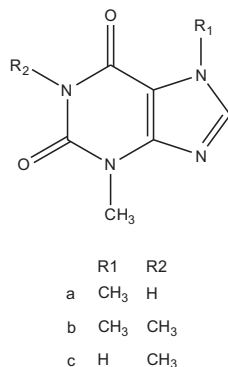*Corresponding author; E-mail: branco@uefs.br

	R1	R2
a	CH$_3$	H
b	CH$_3$	CH$_3$
c	H	CH$_3$

Fig. 1. Chemical structure of theobromine (a) and related compounds (b and c)

'energy' dietary supplements, including transdermal patches, supplemental products that are used for weight loss and energy drinks. Although herbal medicines are often perceived as being natural and therefore safe, they are not free from adverse effects. Adulteration, substitution, contamination, poor identification, lack of standardization, incorrect preparation and/or dosage and inappropriate labelling are the most common problems with medicinal herbs (*11,12*). In this context, a control of methylxanthine content has been instituted by many governmental/legal authorities, including the International Olympic Committee (IOC). The development of easy-to-use, sensitive and specific methods for the determination of methylxanthines in biological fluids and solid and liquid dietary supplements has become necessary (*9,13*).

The theobromine biosynthetic pathway consists of the methylation of xanthosine by *S*-adenosyl-L-methionine (SAM)-dependent 7-N-methyltransferase (EC 2.1.1.158), followed by a second step that involves a nucleosidase which catalyses the hydrolysis of 7-methylxanthosine by N-methylnucleosidase (EC 3.2.2.25) (Fig. 2). The last step of theobromine synthesis is also catalysed by a SAM-dependent N-methyltransferase called theobromine synthase (EC 2.1.1.159). This enzyme is different from the enzyme that catalyses the first step in the pathway
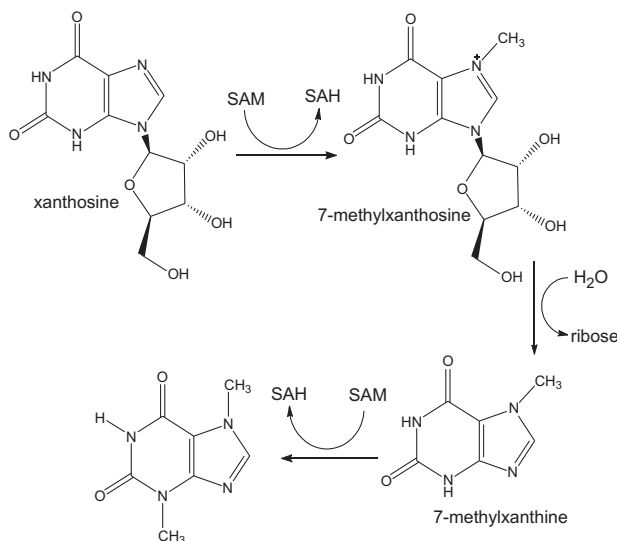
because it is specific for the conversion of 7-methylxanthine to theobromine (*4*). The first enzyme in the pathway can initiate the biosynthesis of both caffeine and theobromine.

Theobromine is the major alkaloid in *Theobroma cacao*. Its seed contains between 1 and 4 % of this compound; it is therefore a plant with tremendous economical importance because of its use in beverages and chocolates (*3,7,14*). The quantity and composition of theobromine in plants can be influenced by different factors such as genetic variability, environmental conditions, age, time of collection, processing and preparation of drugs (*15,16*).

Theobromine (Fig. 1a) and its related compounds (Figs. 1b and 1c) are considered beneficial because they show pharmacologically significant positive effects. These effects include the stimulation of various bodily systems (*17*). The action of theobromine on the central nervous system (CNS) is generally considered weak or nonexistent, while a few studies investigating its effects reported that it acts mainly as a diuretic and a bronchial smooth muscle relaxant (*3,9*). On the other hand, the consumption of high concentrations of theobromine-related compounds, mainly caffeine, may cause cardiac arrhythmia, excitement, nausea, gastritis, cancer, malfunction of the kidneys and asthma (*18,19*).

Food is the most important exposure route of humans to theobromine-related compounds. Hence, food is considered to play an important role in methylxanthine toxicity, not only in a direct way by providing large doses of caffeine and theobromine in beverages or chocolate, but also by accelerating the dissolution and absorption rates of theophylline from sustained release of theophylline preparations (*20,21*). For example, theobromine and theophylline are prohibited in animal feed because of their lower rate of metabolism, while caffeine is considered a drug of abuse in humans when found in concentrations higher than 12 mg/mL in urine (*13,22*). This fact has led to increased interest in developing reliable methods for the assessment of theobromine-related compounds in different biological matrices (*23,24*). Different analytical techniques such as high-performance liquid chromatography (HPLC) and spectrophotometric methods are used to determine methylxanthines in food, beverages, pharmaceutical preparations and biological fluids (*23–33*). In this context, reversed phase high-performance liquid chromatography performed with a UV detector (RP-HPLC) is the most commonly used technique to analyse theobromine-related compounds because of its efficiency, sensitivity, specificity and speed.

The quantification of each compound in a mixture in a single chromatographic run has been extensively studied in the literature (*34,35*). It is therefore of great interest not only for the food industry, but also for the pharmaceutical sectors, to develop more sensitive and accurate procedures to quantify theobromine-related compounds in natural and processed products and in biological fluids.

Other detectors commonly associated with RP-HPLC have also been used to analyse these compounds. The greatest breakthrough was the coupling of HPLC to mass spectrometry (MS). This combination allows the simul-

Fig. 2. Biosynthesis of theobromine

taneous examination of nonvolatile compounds through their mass spectra, which provides information on the chemical structure of unknown compounds (*36*). MS is an analytical technique that identifies the chemical composition of a sample on the basis of the mass-to-charge ratio of charged ions. The technique has both qualitative and quantitative uses.

A new method based on near infrared spectroscopy (NIRS) was validated and compared to high-performance liquid chromatography coupled with UV (HPLC-UV) and an electrospray ionization with quadrupole ion trap mass spectrometry detector (HPLC-ESI-MS/MS). The detection, identification and quantification of theobromine were then made by characteristic patterns of fragmentation. The detection limits were found to be 0.244–0.60 ng per 100 g for HPLC (more sensitive) and 0.05 g per 100 g for spectroscopy (*31*). Electrospray ionization (ESI) is an alternative technique that allows the transfer of ions from a solution to the gas phase for analysis by mass spectrometry. ESI-MS was first used in the ionization of intact chemical species but now has found wide acceptance in the identification of large biologically important molecules (*37,38*).

Analysis of Theobromine and Related Compounds by RP-HPLC/UV

Food

Analysis of theobromine and caffeine in different brands of cocoa products is described in Table 1 (*6,14, 39–41*). The average amount of these compounds obtained by Caudle *et al.* (*14*) was 5.1 mg per 100 g; the precision and accuracy were compared between the method of the Association of Official Analytical Chemistry (AOAC International), approved for the quantification of methylxanthines, and an aqueous extraction using the standard addition method. The AOAC method for quantification can be performed with both internal and external standards. The recovery of theobromine was

99.6 % using the standard addition method, but only 89.3 % using the AOAC International method. Although the standard addition method is expected to be more precise and accurate, it does not require the use of organic solvents and is less developed in stages, so the solid content of methylxanthines in chocolate may not be correctly determined.

Ramli *et al.* (*39*) determined the levels of theobromine in 32 samples of popular brands of local (Kuala Lumpur, Malaysia) and imported chocolates. In the local chocolate, the mean titre was 0.72 mg/g in milk chocolate and 0.85 mg/g in dark chocolate. The amount of theobromine in white chocolate was below 0.05 mg/g. In imported chocolate, the mean level was 1.05 mg/g in dark chocolate, 0.76 mg/g in milk chocolate and 0.74 mg/g in white chocolate. The mean values in chocolate coating and chocolate coating made from fat substitute were 0.82 and 0.49 mg/g, respectively. Meanwhile, Meng *et al.* (*40*) obtained 8.83 and 1.26 mg/g in commercial dark and milk chocolate, respectively. The amount of theobromine in white chocolate was below the detection limit.

Beverages

The routine determination of the quality of tea has recently gained substantial importance due to its pharmacological effect and its application in the food industry. Table 2 (*19,23,24,34,42–50*) summarises these data.

Genarro and Abrigo (*23*) analysed theobromine using reversed phase ion-interaction HPLC/UV as a strategy for spectrophotometric detection. The interaction reagent used in the mobile phase was octylamine orthophosphate, and the level of detection for theobromine using this method was found to be 0.15 ppm. In addition, Meyer *et al.* (*24*) used RP-HPLC to analyse drinks with amperometric detection. The detection limit for theobromine was 2.5 ng. Furthermore, Nakakuki *et al.* (*42*) changed the previous method by switching the type of detection (UV), the mobile phase and adding a

Table 1. HPLC of theobromine and related compounds in food

Analyte	Sample	Column (L, D, P)	Mobile phase	Stationary phase and wavelength	Ref.
Isocratic					
caffeine and theobromine	chocolate cereals	15, 0.46, $5 \cdot 10^{-4}$	MeCN/H_2O (10:90) adjusted to pH=3 with H_3PO_4	ODS-3 100 Å C_{18}; UV at 278 nm	(*6*)
caffeine and theobromine	non-chocolate methyl-xanthine-spiked and chocolate cereal products	15, 0.46, $5 \cdot 10^{-4}$	MeCN/H_2O (10:90) adjusted to pH=3 with H_3PO_4	ODS-3 100 Å C_{18}; UV at 278 nm	(*14*)
caffeine and theobromine	chocolate couverture and chocolate coal	30, 0.40, $10 \cdot 0^{-4}$	MeOH/CH_3COOH/H_2O (20:1:79)	μ-Bondapak 10 μm; UV at 278 nm	(*39*)
Gradient					
phenolic acids and theobromine	dark, milk and white chocolate	25, 0.46, $5 \cdot 10^{-4}$	A: 0.1 % TFA in MeCN, B: 0.1 % TFA in H_2O 0–10 % A, 5 min; 10–25 % A, 25 min; 25–100 % A, 6 min	C_{18} reversed phase*	(*40*)

L: length (cm), D: diameter (cm), P: particle size (cm), ODS: octadecylsilyl
*wavelength not listed, but article refers to Natsume *et al.* (*41*)

Table 2. HPLC of theobromine and related compounds in beverages

Analyte	Sample	Column (L, D, P)	Mobile phase	Stationary phase and wavelength	Ref.
Isocratic					
theobromine-related compounds	tea and coffee	24, 0.44, $5 \cdot 10^{-4}$	H_2O/EtOH/CH_3COOH (75:24:1, by volume)	100 RP-18; UV at 273 nm	(19)
theobromine-related compounds	coffee, tea and cola beverages	25, 0.46, $5 \cdot 10^{-4}$	octylamine orthophosphate	100 RP-18; UV at 274 nm	(23)
adenine and theobromine--related compounds	coffee, tea and cacao	–	phosphate buffer adjusted to pH=3.5/MeOH (90:30, by volume)	C_{18} reversed phase; amperometric detection	(24)
methylxanthines	tea	15, 0.40, $5 \cdot 10^{-4}$	1 % CH_3COOH/MeCN (95:5, by volume)	ODS-3; UV at 273 nm	(34)
caffeine and theobromine	tea	25, 0.46, $5 \cdot 10^{-4}$	H_2O/MeCN/MeOH/H_3PO_4 (82.5:11:6:0.5, by volume) (40 °C)	C_{18} UG-120 Å; UV at 272 nm	(42)
theobromine-related compounds	chocolate, coffee, tea, coconut water	15, 0.40, $5 \cdot 10^{-4}$	EtOH/H_2O/CH_3COOH (20:75:5, by volume)	C_{18} reversed phase; UV at 273 nm	(43)
catechins and xanthines	tea	25, 0.40, $5 \cdot 10^{-4}$	MeCN/0.1 % H_3PO_4 in water (by volume)	C_{18} reversed phase; UV at 210 nm	(44)
theobromine-related compounds	natural water	15, 0.39, $4 \cdot 10^{-4}$	MeOH/H_2O (80:20, by volume) adjusted to pH=2.5 with hydrochloric acid	C_{18} reversed phase; UV at 272 nm	(45)
methylxanthines	tea, soft drinks and coffee	5, 0.2, $2 \cdot 10^{-4}$	MeCN/H_2O (5:95, by volume)	C_{18} reversed phase; UV at 274 nm	(46)
Gradient					
theobromine-related compounds	black tea and mate tea	25, 0.46, $5 \cdot 10^{-4}$	A: H_2O, B: MeOH	RP-18 ODS-3; UV at 273 nm	(47)
polyphenols and methylxanthines	tea	25, 0.46, $5 \cdot 10^{-4}$	A: 3 % CH_2O_2, B: MeOH 2–32 % B, 20 min; 40 % B, 30 min; 95 % B, 40 min	C_{18} reversed phase; UV between 200 and 400 nm	(48)
methylated catechins, purine alkaloids and gallic acid	tea	15, 0.46, $5 \cdot 10^{-4}$	A: CH_2O_2 (pH=2.5), B: MeOH (40 °C) 82–40 % A, 18–60 % B, 0–15 min	ODS-100 Z; UV at 280 nm	(49)
catechins, gallic acid, strictinin, caffeine and theobromine	tea	15, 0.46, $5 \cdot 10^{-4}$	A: 0.25 % H_3PO_4/MeCN (20:1, by volume pH=2.4), B: A/MeOH (5:1, by volume, pH=2.5) 10 % B, 0–10 min; 10–50 % B, 10–20 min; 50–95 % B, 20–30 min; 95 % B, 30–65 min	C_{18} reversed phase; UV at 210 nm	(50)

L: length (cm), D: diameter (cm), P: particle size (cm), ODS: octadecylsilyl

pre-column (10 cm×4.6 mm), packed with polyvinylpoly-pyrrolidone. This method allowed detection of theobromine in less than 10 min (retention time). For a standard solution of theobromine using this method, the relative standard deviation (RSD) was about 0.3 % for the retention time and about 2.5 % for the peak area. In addition, the calibration curve of theobromine was linear from 5 to over 1000 ng.

Lacerda *et al.* (*47*) evaluated commercial samples of black tea, mate tea and other types of tea using different extraction methods: decoction, ultrasonic and microwave treatments. The authors used a Nova-Pak C_{18} pre-column, an analytical RP-18 LiChospher column (Alltech Inc.,

Springfield, KY, USA) and an external standard to obtain a good correlation coefficient to theobromine (0.9998). The microwave-assisted extraction appeared to be more efficient than other extraction methods. Unfortunately, the retention time and detection limit were not listed.

Horžić *et al.* (*48*) compared the content of theobromine in teas and herbal infusions (*Camellia sinensis* L.) as well as the effect of different extraction conditions (water temperature and number of extractions). According to the results, the content of theobromine increased with water temperature (60<80<100 °C) and decreased with the number of repeated extractions (1st extraction>2nd extraction>3rd extraction). This analysis exhibited an

average 3.8-fold difference between the 1st and 3rd extracts of all teas, while the subsequent extracts of herbal infusions showed almost negligible content of theobromine. In addition, Hu *et al.* (*49*) also examined teas of *C. sinensis* but prepared samples through infusion in two ways: 50 % acetonitrile solution (method A) and boiling in distilled water (method B). The concentration of theobromine was higher in tea infusions prepared by method A.

Bispo *et al.* (*43*) determined the concentration of theobromine-related compounds in beverages in runs of only 6 minutes. In this study, the calibration curve for theobromine had good linearity (0.9996) and a relative standard deviation of 0.64 %. Their concentrations ranged from 0.1 pg/mL to 32 mg/mL. The method proved to be appropriate and required no derivatisation of the samples.

Mizukami *et al.* (*50*), using catechol as an internal standard, analysed theobromine-related compounds, catechins, gallic acid and strictinin in commercial tea. According to the authors, this was the best alternative because it was cheaper, especially when there were multiple compounds to be analysed in a single sample. The method offered good repeatability, reproducibility, recovery rates and component resolution. The addition of ascorbic acid preserved the stability of the expensive catechin reference standard in the stock solution for 1 year when stored at –30 °C. Only traces of theobromine were identified in the sample. The detection limit, correlation coefficient and limit of quantification for the calibration curve were 0.44, 0.998 and 1.35 μg/mL, respectively. The range was from ND (not detected) to 10 μg/mL with a mean of 9 μg/mL, and from 7 to 19 μg/mL with a mean of 13 μg/mL in the bottled and brewed tea, respectively.

Alves and Bragagnolo (*34*) optimised the methodology for the analysis of tea, creating a method for the determination of caffeine in coffee using HPLC. The method showed good correlation coefficients for the calibration curve, good recovery and a good limit of detection for theobromine (0.99991, 95 % and 0.0003 g per 100 g, respectively). The process proved to be simple, economical and precise.

De Aragão *et al.* (*19*) used full factorial multivariate analysis in three levels for the optimisation of chromatographic separation of theobromine-related compounds. The mobile phase was studied in terms of the polarity, flux, selectivity and acidity. The resulting method had high resolution for all methylxanthines in less than 6 min, and the reported detection limit for theobromine was 0.07 g/L. The optimisation was fast, and extraction or derivatisation of the samples was not required.

In the work by Sharma *et al.* (*44*), the effects of the method of elution, mobile phase, wavelength and temperature of the column in the separation of theobromine-related compounds were studied. The optimum developed method had good linearity, with a correlation coefficient ranging from 0.954 to 0.990, good reproducibility and accuracy. Furthermore, it showed satisfactory results and could be applied to any type of tea for routine analysis.

Da Costa Silva and Augusto (*45*) first used solid phase extraction to analyse natural water. Organically

modified silica (ORMOSIL) that was molecularly imprinted was subsequently prepared through a simple sol-gel procedure and evaluated as a specific sorbent for solid-phase extraction (SPE) of methylxanthines from a water sample. Caffeine was used as a template for comparison of molecularly imprinted ORMOSIL with non-imprinted silica (NIS) and SPE C_{18} cartridges. The molecular imprinting technique was found to be capable of producing materials with high selectivity for a given compound.

Printed silica prepared by the sol-gel method is produced by the incorporation of template chains of organically modified silica (ORMOSIL). Because of the specific nature of the interaction between the molecularly imprinted materials and selectable molecules, they have been employed in several analytical techniques, including liquid chromatography (*51,52*). For example, da Costa Silva and Augusto (*45*) reported that molecularly imprinted silica obtained one peak that was identified as theobromine (retention time of 5.05 min). The peaks in the chromatogram using non-imprinted silica were noticeably minor, confirming the advantage of molecular imprinting. Therefore, molecularly imprinted ORMOSIL was highly specific, demonstrating its good selectivity with a detection limit of 0.09 mg/L and a limit of quantification of 0.29 mg/L for theobromine.

The application of the new narrow-bore monolithic column for simultaneous determination of methylxanthines in various real samples such as soft drinks, tea and coffee was also investigated (*46*). The separation was optimized and validated. The proposed method offered shorter analysis time and drastic reduction in the consumption of mobile phase and organic solvents.

Biological fluids

The chromatographic conditions for the analysis of theobromine-related compounds in biological fluids are described in Table 3 (*25,26,29,30,43,53*). Pérez-Martínez *et al.* (*26*) used reversed phase high-performance liquid chromatography with UV detector (RP-HPLC/UV) for the determination of methylxanthines in urine by using a micellar mobile phase. One advantage is that this method does not require the inclusion of a procedure for prior cleaning of the sample (*28*). RP-HPLC then separates these molecules in biological fluids on the basis of differences in their hydrophobicity. More specifically, the components of the analyte mixture flow over stationary-phase particles bearing pores large enough for them to enter, where interactions with the hydrophobic surface removes them from the flowing mobile-phase stream. The strength and nature of the interaction between the sample particles and the stationary phase depend on both hydrophobic and polar interactions. The authors also used a guard column (35×4.6 mm) with characteristics similar to the analytical column and a flow rate of 1 mL/min. The composition of the appropriate mobile phase (pH, concentration of SDS, nature and concentration of organic solvents) for separation was also investigated. The detection limit with UV for theobromine was 0.4 mg/mL, and the procedure allowed the determination of three compounds in the sample in less than 10 min (*26*).

Zambonin *et al.* (*29*) used RP-HPLC to analyze theobromine in human urine samples with a diode array de-

Table 3. HPLC of theobromine and related compounds in biological fluids

Analyte	Sample	Column (L, D, P)	Mobile phase	Stationary phase and wavelength	Ref.
Isocratic					
theobromine-related compounds	human urine and plasma	15, 0.03, $5 \cdot 10^{-4}$	precolumn: 17 M/glycerol/H_2O (0.1:0.5:99.4) column: 17 M $CH_3COOH/C_3H_5(OH)_3/MeOH/H_2O$ (0.5:0.5:10–99:89–0)	ODS-HG-5; UV/EM at 273 nm	(25)
theobromine-related compounds	urine	12, 0.46, $5 \cdot 10^{-4}$	micellar: aqueous solutions of SDS/MeOH, C_3H_7OH or $C_5H_{11}OH$ adjusted to pH=3–7 with a 0.01 M phosphate buffer	ODS-2 C_{18}; UV at 273 nm	(26)
methylxanthine	urine	25, 0.21, $5 \cdot 10^{-4}$	MeOH/buffer (20:80), buffer: 5 mM $C_6H_8O_7$ adjusted to pH=5.0 with triethylamine	LC$_{18}$-DB; UV/DAD at 280 nm	(29)
theobromine-related compounds, paraxanthine and nicotine	human milk	25, 0.46, $5 \cdot 10^{-4}$	MeOH/buffer (20:80), buffer: 5 mM sodium octane sulphonate, 10 mM $C_6H_8O_7$ adjusted to pH=5.8 with triethylamine	LC$_{18}$-DB; UV/DAD at 260 nm	(30)
TRC	urine	15, 0.40, $5 \cdot 10^{-4}$	EtOH/H_2O/CH_3COOH (20:75:5)	C_{18} reversed phase; UV at 273 nm	(43)
Gradient					
theobromine and caffeine	saliva, plasma and urine	5, 0.21, $17 \cdot 10^{-4}$	A: 0.1 % (by volume) CH_2O_2 in double-distilled water, B: MeCN 2 % B, 0–0.5 min; 10 % B, 0.5–0.7 min; 13 % B, 0.7–1.25 min; 14 % B, 1.25–1.5 min; 50 % B, 1.5 min	C_{18}; UV/DAD at 280 nm	(53)

L: length (cm), D: diameter (cm), P: particle size (cm), ODS: octadecylsilyl

tector (DAD) and a pre-column (20×2.1 mm, 5 μm) at a flow rate of 0.2 mL/min. The recovery, limit of detection and quantification for theobromine were (99.3±6.3) %, 0.3 and 1.2 mg/mL, respectively. Additionally, Aresta *et al.* (*30*) analysed theobromine in human milk, but changed the composition of the buffer, the size of the column/ pre-column and the flow (1 mL/min). A recovery of 60.2 was subsequently observed for theobromine.

A diode array detector consists of a single integrated circuit that has a radiation sensor, a charge storing capacity and a reading unit. The overall performance of a device with a DAD is determined largely on the characteristics of the detector, such as spectral response range, accuracy and precision in the measurements of wavelength and light intensity, resolution, sensitivity, and therefore signal/noise and band dynamics. It is capable of generating a relatively large number of data points in a very short period of time by scanning the wavelength range (*54*).

Bispo *et al.* (*43*) analyzed caffeine, theobromine and theophylline in urine following the same conditions as above (*29*) at concentrations ranging from 0.1 pg/mL to 13.2 μg/mL.

Analysis in urine was performed under optimised conditions mentioned above by de Aragão *et al.* (*19*) and da Costa Silva and Augusto (*45*); the latter authors concluded that the efficiency of theobromine extraction using imprinted silica was low, approx. 68 %. These numbers were even smaller for methylxanthines before ingestion of milk chocolate. The chromatograms of extracts from

C_{18} extraction showed several peaks undetected in the chromatograms of molecularly imprinted ORMOSIL. Also, the peak of theobromine in the C_{18} chromatogram was misshapen. According to the authors, this observation was the result of a co-retained analyte not present in the extract of molecularly imprinted ORMOSIL.

Finally, Hieda *et al.* (*25*) analysed theobromine-related compounds in urine and human plasma by HPLC with bombardment of atoms of 'frit-fast' type (RP-HPLC- -frit-FAB-MS). The authors used a pre-column (ODS- -HG-15/30 35×0.3 mm, 5 μm) and two mobile phases. Theobromine was observed as a pseudo-molecular ion [M+H]$^+$ at *m/z*=181, but little fragmentation was apparent. From Table 3, it can be seen that all tests were performed under isocratic conditions.

Ptolemy *et al.* (*53*) combined a single ultracentrifugation-based sample pretreatment and liquid chromatography-tandem mass spectrometry to quantify theobromine and caffeine in saliva, plasma and urine samples. The assay was linear over a 160-fold concentration range from 2.5 to 40 μmol/L for both theobromine (average R^2=0.9968) and caffeine (average R^2=0.9997).

Plants

Reginatto *et al.* (*16*) analysed theobromine-related compounds in species of the genus *Ilex*. A pre-column RP-C_{18} (39×3.0 mm, 5 nm) and a flow rate of 0.5 mL/min were used. Interestingly, these compounds were only found in two varieties of mate (*Ilex paraguariensis*). However, the procedure employed had the advantages of being simple, precise and accurate (Table 4; *15,16,32,55–63*).

Table 4. HPLC of theobromine and related compounds in plants

Analyte	Sample	Column (L, D, P)	Mobile phase	Stationary phase and wavelength	Ref.
Isocratic					
theobromine-related compounds	cocoa beans	15, 0.39, $4 \cdot 10^{-4}$	20 % MeOH/H_2O (by volume)	C_{18} reversed phase; UV at 274 nm	(15)
theobromine-related compounds	*Ilex* species	15, 0.39, $5 \cdot 10^{-4}$	MeOH/H_2O (25:75)	RP-C_{18}; UV at 280 nm	(16)
theobromine	*Paullinia cupana*	25, 0.46, $5 \cdot 10^{-4}$	MeOH/H_2O (70:30) adjusted to pH=3.5 with H_3PO_4	C_{18} reversed phase; UV/DAD at 254 nm	(32)
caffeine and theobromine	*Ilex paraguariensis*	25, 0.40, $5 \cdot 10^{-4}$	MeOH/H_2O (4:6)	CLC-ODS (M) RP-18 UV at 280 nm	(55)
caffeine and theobromine	*Ilex paraguariensis*	25, 0.46, $5 \cdot 10^{-4}$	MeOH/H_2O (75:25) (40 °C)	ODS UV at 272 nm	(56)
methylxanthines and phenolic compounds	*Ilex paraguariensis*	25, 0.46, $5 \cdot 10^{-4}$	MeCN/0.1 % CH_2O_2 (15:85, by volume)	C_{18} reversed phase; UV/DAD at 280 nm	(57)
Gradient					
caffeine, theobromine and phenolic compounds	progenies of *Ilex paraguariensis*	25, 0.46, $5 \cdot 10^{-4}$	A: 0.3 % CH_3COOH/H_2O, B: MeOH 15–20 % B, 0–20 min; 20–85 % B, 20–25 min; 85 % B, 25–30 min	LC-18; UV at 265 nm	(58)
methylxanthines, caffeoyl, derivatives and flavonoids	*Ilex paraguariensis*	25, 0.46, $5 \cdot 10^{-4}$	A: H_2O/CH_3COOH (98:2, by volume), B: MeOH/CH_3COOH (98:2, by volume) 17 % B to 20 % B, 10 min; 20 % B (isocratic), 5 min; 20 % B to 23 % B, 10 min; 23 % B to 100 % B, 5 min	RP-C_{18}; UV/DAD at 273 nm	(59)
catechins and methylxanthines	*C. sinensis*, *C. ptilophylla* and *C. assamica* var. *kucha*	15, 0.46, $5 \cdot 10^{-4}$	A: 5 % MeCN/0.05 % H_3PO_4 (85 %), B: 50 % MeCN/0.05 % H_3PO_4 (85 %) 90 % A and 10 % B, 0–7 min; 10–15 % B, 7–10 min; 15–70 % B, 12–20 min	RP-18 DAD at 231 nm	(60)
phenolic compounds, theobromine-related compounds, theacrine and theanine	*C. sinensis*, *C. ptilophylla*, *C. assamica* and *C. assamica* var. *kucha*	15, 0.46, $5 \cdot 10^{-4}$	A: 85 % H_3PO_4/H_2O (0.05:99.95), B: MeCN 2 % B, 0–4 min; 2–9 % B, 4–21 min; 9–22 % B, 21–32 min; 23 % B, 32–45 %	RP-amide C_{16}; DAD between 210 and 280 nm	(61)
polyphenols and purine alkaloids	leaves of 22 tea cultivars	25, 0.46, $5 \cdot 10^{-4}$	A: 1 % CH_2O_2/H_2O (by volume, 1:1), B: MeCN or MeOH 4–25 % B, 0–60 min	RP-C_{18}; UV/DAD at 275 nm	(62)
purine alkaloids and phenolic compounds	*Cola* sp. and *Garcinia kola*	25, 0.40, $5 \cdot 10^{-4}$	A: 2 % CH_3COOH/H_2O, B: MeCN/H_2O/concentrated CH_3COOH (4:9:1) 90 % A and 10 % B, 0–8 min; 90 % A and 10 % B, 8–38 min; 77 % A and 23 % B, 38–50 min; 60 % A and 40 % B, 50–70 min; 10 % A and 90 % B, 70–73 min; 10 % A and 90 % B, 73–78 min; 90 % A and 10 % B, 78–93 min	C_{18} reversed phase; DAD at 280 nm	(63)

L: length (cm), D: diameter (cm), P: particle size (cm), ODS: octadecylsilyl

An analysis by Gnoatto *et al.* (*55*) also aimed to compare seven extractive methods on *Ilex paraguariensis* and determine the influence of extraction conditions (in a Soxhlet extractor and by decoction) on methylxanthine yield. The limits of detection and quantification of theobromine were 0.09 and 0.30 g/mL, respectively, assessed within the linearity for the method (0.32–4.85 g/mL). Extraction of theobromine by decoction with acidic aqueous

solution showed higher efficiency. Therefore, for concomitant theobromine and caffeine quantification the decoction with acidic aqueous solution was recommended.

Lopes *et al.* (*56*) determined theobromine and caffeine in young and old plant leaves. The levels of theobromine and the coefficient of variation found in young and old leaves were 0.05 and 0.08 %, and 3.61 and 1.16 %, respectively. The employed methodology proved to be suitable for studies of quality control and/or adulteration.

The content of methylxanthines in 16 mate progenies from 4 regions in Brazil was evaluated by Cardozo Jr. *et al.* (*58*). The peaks in the chromatograms were identified by comparison with the retention times of standards. Significant differences were observed in the content of theobromine in 16 progenies, dividing them into 3 groups (≤0.086, ≥0.091 and 0.237 %), and among the 4 regions of origin. No difference was found among the three localities where the progenies were grown.

The effect of light intensity on the content of methylxanthines in mate was also investigated (*64*). Comparing the content of theobromine in plants exposed to 100 and 32 % sunlight, a significant difference between the treatments was observed: a higher content of methylxanthine was found in mate grown under 32 % sunlight. In relation to natural shading produced by other trees, the theobromine content was not statistically significant in plants grown under 93, 41 and 5 % of sunlight. However, a negative correlation was found between the accumulated biomass and the content of methylxanthines. These results suggest that moderate shade does not change the concentration of methylxanthines and the accumulated biomass.

Pagliosa *et al.* (*57*) compared the determination of methylxanthines, phenolic compounds and antioxidant activity in mate leaves and bark (residual biomass) in both aqueous and methanolic extracts. There was no significant difference (p>0.05) between the theobromine content of mate leaves (0.56 mg/100 g) and bark (0.30 mg/100 g).

The compound variation during yerba mate processing (harvesting, roasting or zapecado, drying, natural aging and forced aging) was investigated by Isolabella *et al.* (*59*). According to López *et al.* (*65*), the industrialization process can modify the qualitative and quantitative composition and the pharmacological activities. The results showed an increase in the content of theobromine after roasting when compared to the green leaves. There was no significant difference (p>0.05) between the theobromine content of roasted, dried, forced to age and naturally aged yerba mate.

Yang *et al.* (*60*) analyzed both methylxanthines and catechins in species of the genus *Camellia* by RP-HPLC-DAD. The method showed good correlation coefficient, level of detection and recovery rates. The study reported 2.7 % of theobromine in *C. sinensis*, 4.85 % in *C. ptilophylla* and 0.45 % in *C. assamica* var. *kucha*. In addition, Peng *et al.* (*61*) used an amide-C_{16} column equipped with a guard column (4×20 mm, 5·10⁻⁴ cm) using UV/DAD at 210 nm and obtained good results. The method was thus validated and used for analysis. Linearity, correlation coefficient, retention time, limit of detection and limit of quantification for theobromine were (0.01–1)·10⁻³ mg, 0.9998, 12.6, 0.3 and 0.9 ng, respectively. The content of theobromine in *C. sinensis*, *C. assamica*, *C. ptilophylla* and *C. assamica* var. *kucha* were subsequently found to be (0.01±0.01), (0.24±0.01), (4.00±0.12) and (0.08±0.01) ng, respectively. The method was efficient and allowed for the complete separation of all compounds.

Wang *et al.* (*62*) used HPLC-DAD-ESI-MS/MS with a guard column (C_{18}, 4×2.0 mm) for scanning in a range from *m/z*=50 to 1500. The identification of methylxanthine in the Guihuaxiang cultivar, showed a pseudo-molecular ion [M+H]⁺ of *m/z*=181, which had maximum absorption at two wavelengths (240 and 270 nm). The method was also completely validated.

Niemenak *et al.* (*63*) analyzed the content of theobromine in mature seeds of *Cola* sp. and *Garcinia kola* in order to ascertain the genetic relationship within and between taxonomic populations. The analysis was performed on a column maintained at 26 °C with a guard column (LiCroCART® 4-4 LiChrospher 100 RP-18; 5 mm, Merck Chemicals, Sao Paolo, Brazil) and theobromine was detected in all accessions studied and was the most abundant alkaloid in *C. acuminata* from Okala, Gabon (1277 mg/kg), in *C. nitida* from Muyuka and Buea, Cameroon (1570 and 1550 mg/kg) and in *C. anomala* from Bamenda, Cameroon (3370 mg/kg).

Brunetto *et al.* (*15*) used HPLC-UV/DAD with on-line cleaning (solid-phase extraction) of the sample in a dry-packed pre-column (50×4.6 mm i.d.) for the analysis on a ODS-C_{18} (15–40 µm) column (Waters, Milford, MA, USA). This method for determination of theobromine is fast, accurate and sensitive, and can be used in routine analysis of a large number of cocoa samples. The overall performance of a device with DAD is determined largely on the characteristics of the detector, such as spectral response range, accuracy and precision in the measurements of wavelength and light intensity, resolution, sensitivity, and therefore signal/noise ratio and band dynamics. It is capable of generating a relatively large number of data in a very short period of time by scanning the sample in a range of wavelengths (*54*).

Screening of 34 species of *Paullinia* was done by Weckerle *et al.* (*66*) with the aim of verifying the occurrence of purine alkaloids. According to the authors, among the few genera consumed as stimulants, *Paullinia* is the least investigated with respect to chemotaxonomy because of its minor economic impact and low abundance. Peak identification was achieved by comparison with the spectra and retention times of standards. Among the evaluated species, in addition to *P. cupana* and *P. yoco*, which are already recognised as being rich in purines, only *P. pachycarpa* (new) contained theobromine. Specifically, mean values in *P. cupana* ranged from 0.005 to 1.263 % of dry mass. In *P. yoco*, this content varied between different specimens (from 0.000 to 0.438 %), which demonstrated the high variability in theobromine content across wild species.

Sombra *et al.* (*32*) examined theobromine and other compounds in the formulation of tablets containing guarana (*Paullinia cupana*) and compared the results with those obtained from capillary zone electrophoresis (CZE). Elution order, UV spectra, sensitivity and precision were compared between methods. The methods were equivalent in terms of sensitivity, precision and specificity, but

Table 5. HPLC of theobromine and related compounds in biological tissues

Analyte	Sample	Column (L, D, P)	Mobile phase	Stationary phase and wavelength	Ref.
Gradient					
theobromine-related compounds and catechin	pig's ear	25, 0.44, $5 \cdot 10^{-4}$	0.25 M K_3PO_4 adjusted to pH=3.5 with H_3PO_4/MeOH (77:23)	C_{18} reversed phase; UV at 272 nm*	(67)
procyanidins, anthocyanins, theobromine and caffeine	rat tissues (liver, brain, aorta and adipose tissue)	10, 0.21, $18 \cdot 10^{-4}$	A: 0.2 % CH_3COOH, B: MeCN 5 % B to 35 % B, 0–10 min; 35 % B to 80 % B, 10–10.10; 80 % B (isocratic), 10.10–11 min; 80 % B to 5 % B, 11–11.10 min; 5 % B (isocratic), 11.10–12.50 min	binary phase, MS	(68)

L: length (cm), D: diameter (cm), P: particle size (cm)
*wavelength and stationary phase not listed

capillary electrophoresis had a higher efficiency, lower cost per analysis, greater speed, sensitivity and suitable minimal use of organic solvent (44).

Heard *et al.* (67) investigated the effect of *in vitro* transdermal distribution of the major pharmacologically active compounds in the extract of guarana (*Paullinia cupana*), caffeine, theobromine, teophylline and (+)-catechin, through the skin of a pig's ear (Table 5; 67,68). Saturated solutions were prepared in polyethylene glycol (PEG), propylene glycol (PG) and water. The solutions were formulated in a transdermal patch where a dose of 5.55 mg/cm² was considered as ideal. Distribution was determined by the use of a Franz Cell, and RP-HPLC was used to quantify the permeability of the studied analytes. For theobromine, the greatest steady state flux was obtained from the water vehicle: $4.50 \cdot 10^{-4}$ mg/(cm²·h) with ~$9.81 \cdot 10^{-3}$ mg/cm² permeating after 24 h. The steady state flux from the PEG vehicle was $5.10 \cdot 10^{-6}$ mg/(cm²·h) with ~$6.74 \cdot 10^{-3}$ mg/cm² permeating after 24 h, from the PG vehicle was $8.38 \cdot 10^{-5}$ mg/(cm²·h) with ~4.09 µg/cm² permeating after 24 h, and from the 5.55 mg/(cm²·cm²) patch was 0.076 µg/(cm²·h) with ~1.36 µg/cm² permeating after 24 h. This study established that the simultaneous transdermal rate of permeation is highly dependent on concentration and vehicle.

Serra *et al.* (68) investigated procyanidins, anthocyanins, theobromine and caffeine in rat tissues (liver, brain, aorta and adipose tissue) by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, with quadrupole analyzer (UPLC-ESI--MS/MS). The UPLC is a relatively new technology that combines the use of columns with particles smaller than 2 mm and instrumentation that allows operation with high pressures of the mobile phase, which allows significant reduction in the time analysis compared to conventional HPLC (69). The results were obtained 4 h after the administration of a dose corresponding to 1 g of cocoa extract per kg of body mass. The instrumental quality parameters (linearity, detection limit – LOD, and quantification limit – LOQ) were evaluated. The linearity, LOD and LOQ for theobromine were, respectively, 3.3–80, 0.9 and 3.3 nmol/g of fresh tissue in the liver, 4.6–57.5, 1.1 and 4.6 nmol/g of fresh tissue in the brain, 35.8–380, 13.3 and 35.8 nmol/g of fresh tissue in the aorta, and 19.0–227, 5.7 and 19 nmol/g of fresh tissue in the adipose

tissue. The concentration of theobromine at 4 h after the administration of cocoa extract per kg of rat mass was (3.82±0.10) nmol/g in the liver, (25.6±1.42) nmol/g in the brain and (289±6.00) nmol/g in the aorta. The content of theobromine in adipose tissue was not quantified.

Conclusions

This review described the utilization of RP-HPLC/UV in recent years (1992 to 2011) for the determination of theobromine and related compounds in food, beverages, biological fluids and plants. Theobromine is a metabolite belonging to the alkaloid family and its core structure is derived from purine nucleus. This substance is of high economic importance and HPLC is most frequently used for its determination and quantification. Many systems have been developed and optimized for the detection and quantification of theobromine. After comparing many different reports, although the gradient mode is better to analyse these compounds in respect to resolution and speed, the isocratic mode was mostly used due to its practicality. Among the solvents used as mobile phase, a mixture of water/methanol/acetonitrile treated with acetic, phosphoric or formic acid was the most common. Furthermore, RP-HPLC/UV/DAD did not require derivatisation and recording of the absorbance at all wavelengths.

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