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Analytical Techniques Used for Determination of Methylxanthines and their Analogues—Recent Advances

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Methylxanthines (caffeine, theophylline, theobromine) are a popular group of natural purine alkaloids, which are components of many commonly used drugs, parapharmaceuticals and a wide range of food products (e.g. coffee, tea, energy drinks, etc.). Caffeine metabolites (theophylline, paraxanthine, theobromine and other 1-, 3-, 7-methyl tri-, di- and mono-derivatives of xanthine) and hypoxanthine metabolites (xanthine, uric acid) play an important role in the biochemical processes of mammalian organisms. That is why they are the markers of many diseases and are in the focus of the clinicians, pharmaceutical industry and ecologists. In addition to the natural methylxanthines there are structurally related synthetic derivatives: pentoxifylline, dyphylline, xantinol nicotinate and 8-chlorotheophylline that are still valuable pharmaceuticals needing to be monitored. A review of the modern techniques used for determination of methylxanthines for the last 10 years (2000 – March 2010) is presented.

KEYWORDS Xanthine, xanthine metabolites, xanthine determination, methylxanthines, liquid chromatography, capillary electrophoresis

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INTRODUCTION

Methylxanthines (Fig. 1) are heterocyclic organic compounds built from coupled pyrimidinedione and imidazole rings. Those purine alkaloids have been isolated from natural plant materials and include three basic substances: caffeine, theobromine and theophylline.

Caffeine is a 1,3,7-trimethylxanthine with a broad profile of pharmacological action. It acts as a stimulant on the central nervous (CNS) and cardiovascular system, has a mild diuretic properties, and diastolic blood pressure in the smooth muscles (1).

The metabolism of caffeine takes place in the liver, with the participation of cytochrome P450 family. The main metabolites are paraxanthine (84%), theobromine (12%) and theophylline (4%) and to a lesser extent, 1-, 3-, 7-methyl derivatives of uric acid (UA) and xanthine (Fig. 2) (2). That is why caffeine has potential as a metabolic marker substance in humans and more universal metabolic tool in the other species (3–5).

The aim of caffeine analyzing in the pharmaceutical industry is connected with its identification and quantification as active compound of drugs or complex preparations for example with paracetamol (6,7) or acetylsalicylic acid (cold and antitussive preparations) (8).

The largest consumption of caffeine comes from the food industry. Massive intake of this purine implies its quantitative and qualitative determination not only in plant materials as green and black tea (9–14) or guarana (15,16) but in ready-to-eat products, for example beverages (17,18), coffee (18), energizing drinks (19,20) or chocolate (21,22).

The presence of pharmaceuticals (antibiotics, steroids, hormones, analgesics, etc.), their metabolites and other wastewater-derived micropollutants in surface and groundwater is receiving intense public and scientific attention. Taking into consideration the significant effects of caffeine and its wide participation in biological processes due to a still-increasing consumption in various forms, it is postulated that a continuous monitoring of caffeine in environmental material is needed (23,24).

Theophylline (1,3-dimetylxanthine) is the most commonly prescribed bronchodilator, used in the treatment of bronchial asthma and chronic obstructive pulmonary disease. The relaxation effect on the smooth muscle of theophylline is attributed to the inhibition of the phosphodiesterase, with a resulting increase in cyclic adenosine monophosphate (cAMP). Similar to caffeine and theobromine, theophylline is diuretic. However, like the other methylxanthines, theophylline has a narrow therapeutic index; as a result, toxicity can be a significant problem with its chronic use, so careful therapeutic monitoring is essential, especially with other methylxanthines and/or other drugs.

Hypoxanthine and xanthine are formed in the metabolic pathway as a result of the decomposition of adenosine-5'-triphosphate (ATP) into

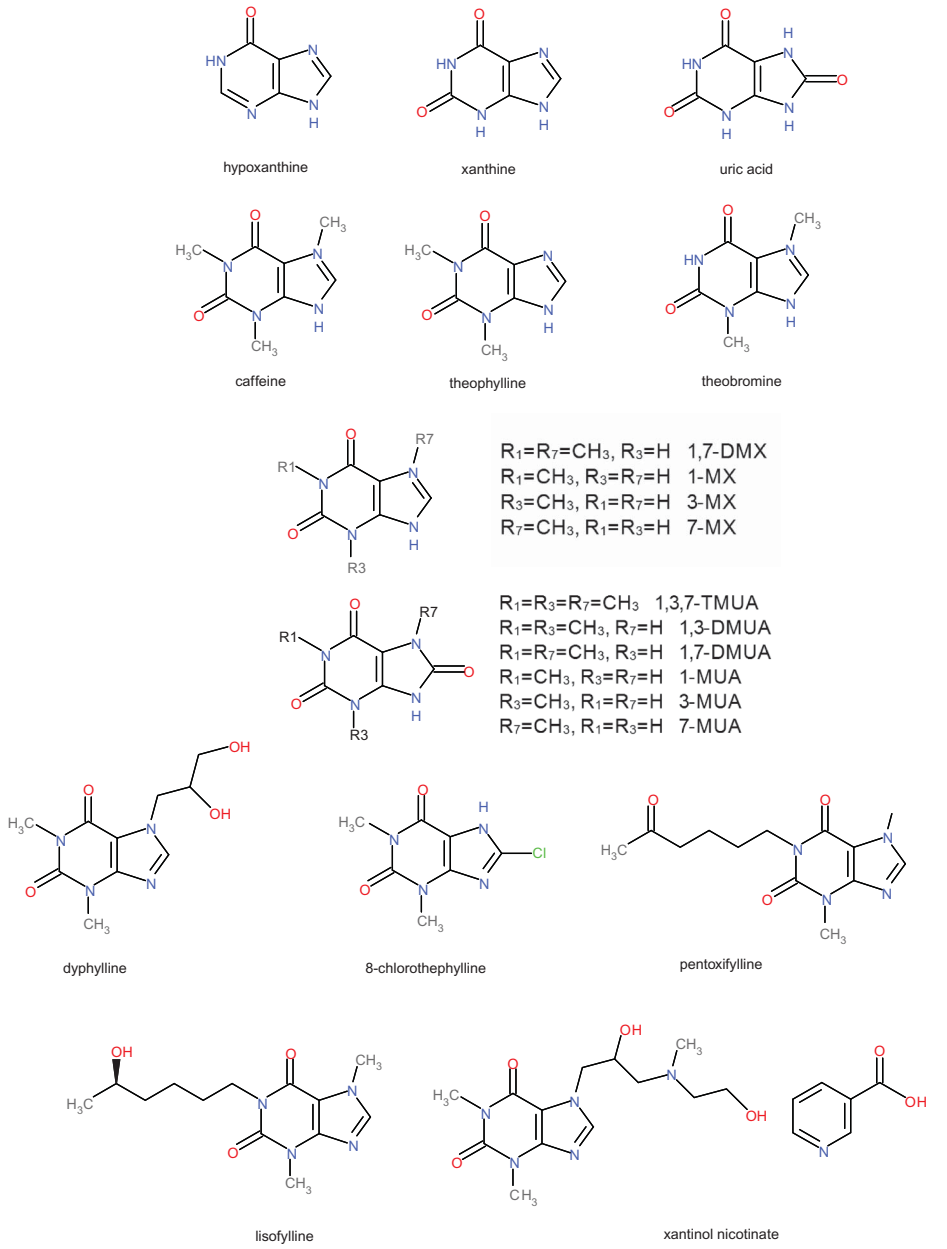

FIGURE 1 The structure of methylxanthines: 1,3,7-TMUA 1,3,7-trimethyluric acid, 1,3-DMUA 1,3-dimethyluric acid, 1,7-DMUA 1,7-dimethyluric acid, 1-MUA 1-methyluric acid, 3-MUA 3-methyluric acid, 7-MUA 7-methyluric acid, 1,7-DMX 1,7 dimethylxanthine, 1-MX 1-methylxanthine, 3-MX 3-methylxanthine, 7-MX 7-methylxanthine (color figure available online).

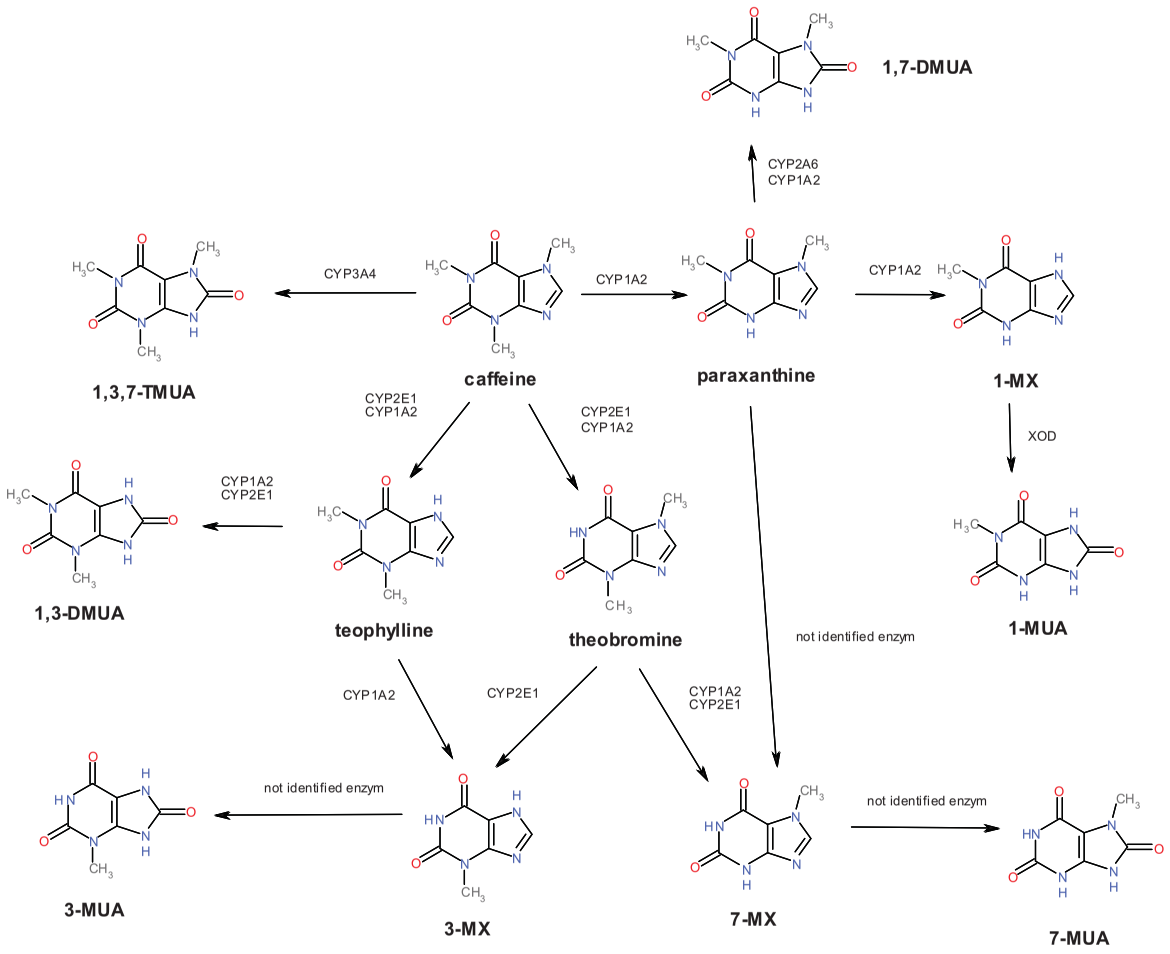

FIGURE 2 The metabolism of caffeine with participation of cytochrome P450 family; 1,7-DMUA 1,7-dimethyluric acid, 1,3,7-TMUA 1,3,7-trimethylouric acid, 1-MX 1-methylxanthine, 1,3-DMUA 1,3-dimethyluric acid, 1-MUA 1-methyluric acid, 3-MUA 3-methyluric acid, 3-MX 3-methylxanthine, 7-MX 7-methylxanthine, 7-MUA 7-methyluric acid (color figure available online).

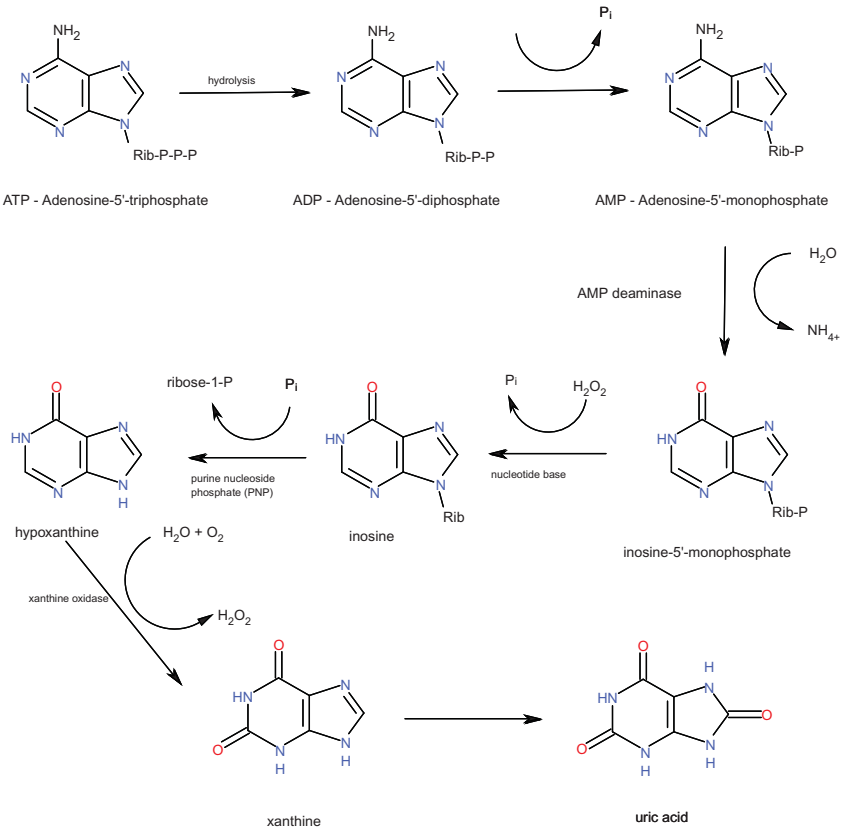

FIGURE 3 The metabolizm of purines; Rib=D-ribose (color figure available online).

adenosine-5'-diphosphate (ADP) and further adenosine-5'-monophosphate, inosine and uric acid (UA) which is the final product of this transformation (Fig. 3).

Increased concentration of any of these compounds results in an elevated UA level that leads to formation and precipitation of monosodium uric crystals in joints, tendons, and surrounding tissues and brings on an acute and painful inflammations (gout) or urolithiasis. Effectively, it may cause kidney damage (25), cardiovascular system diseases (26) and/or insulin metabolism disturbance (diabetes type II) (27). Monitoring concentrations of xanthines in this metabolic path in human tissues and humors (28,29) is also a priority in assessment of other failures connected with cell hypoxia, enzymatic regulatory disturbances or genetic defects (Lysch-Nyhan syndrome (30)) associated with immunological, haematological and neurological dysfunctions.

The synthetic group of methylxanthines in this review concerns dyphylline, 8-chlorotheophylline and xantinol nicotinate, which can be considered as 7- or 8- substituted theophylline analogues and pentoxifylline

with its main metabolite lisofylline (LSF), which can be found as theobromine derivatives.

Pentoxifylline (1-(5-oxohexyl)-3,7-dimethylxanthine) is a hemorheologic agent widely used as a vasodilator in the treatment of peripheral and cerebral vascular disorders. It also acts by reducing blood viscosity that increase blood flow to ischaemic tissues and exerts immunomodulatory effects both in vitro and in vivo in animal models of sepsis. Pharmacokinetic studies have shown that pentoxifylline is transformed into several metabolites (in mammals). One of them: 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (M1) is chiral. Its R enantiomer is known as LSF and has been reported to produce hemorheologic effects similar to pentoxifylline. It is known that from LSF the other metabolites are produced. Recent studies have extended the therapeutic application of LSF especially for diabete mellitus.

Dyphylline is a water soluble derivative of theophylline with broncho- and vasodilator properties. It is used in the treatment of asthma, cardiac dyspnea, and bronchitis.

The compound 8-Chlorotheophylline is one of the components of DMH (Dimenhydrinate), an over-the-counter antiemetic. Like the other xanthines, it is an adenosine antagonist. In this case it acts as a stimulant on the central nervous system and helps in psychomotor and locomotor disorders.

There were no articles concerning analysis or determination of xantinol nicotinate in recent 10 years. This potential drug is under pharmacological and clinical studies with few release results so it will be not found in this review.

All these observations lead to the conclusion that the analysis of natural methylxanthines in pharmaceutical researches, clinical and environmental fields is large and steadily growing due to the fact that they are markers of many dysfunctions and diseases, components of many common medicines, dietary supplements and many commonly used foods as well. The analytical requirements for the development of these compounds is increasing and results in a continuous search for new, reliable, precise, simple, fast and low-cost methods of analysis.

A review of the modern techniques used for quantitative and qualitative determination of methylxanthines with particular emphasis on separation methods especially liquid chromatography (HPLC) and capillary electrophoresis (CE), both with spectrophotometric (UV), mass spectrometry (ESI/MS) and amperometric detection is presented. The literature listing of the last 10 years (2000 - March 2010) was created by searching databases, using the following keywords: HPLC, amperometry, chromatography, electrophoresis and spectrometry associated with: caffeine, theophylline, dyphylline, xanthine, UA, pentoxifylline, lisofylline, 8-chlorotheophylline and xantinol nicotinate. The peculiarities of the analysis of methylxanthines and its metabolites has been discussed and summarized in tabular form

(Tables 1 to 7) including important methodological details. The analyses of biological samples were especially under consideration.

EXTRACTION METHODS

Most papers describe methods applied for the determination of caffeine. Shrivas et al. (17) coupled drop-to-drop solvent microextraction (DDSM) with gas chromatography with mass spectrometry detection (GC/MS). They analyzed the caffeine content of various beverages and food using 7 μL samples. The dichloromethane extraction time of was five minutes. Li et al. (37) proved that subcritical water extraction (SBWE) is an effective extraction fluid not only for caffeine but also for other classes of organic compounds like: chlorophenols, chloro- and methylanilines, nitrotoluenes and polychlorinated biphenyls (PCBs); an on-line coupling system SBWE with HPLC was used.

Theodoridis et al. (33,34) prepared a molecularly imprinted polymer (MIP) with caffeine as the template molecule. In their work the authors successfully applied extraction protocol to the direct extraction of caffeine from beverages and spiked human plasma (34) and described an automated sample preparation scheme that allowed the on-line extraction of a sample prior to HPLC analysis utilizing a low-pressure system (33).

Selective extraction of theophylline from human serum samples was investigated by Khorrami et al. (32). The authors presented the technique based on the simultaneous forward-extraction of analyte from aqueous sample to an organic solvent and back-extraction to MIP solid phase. Conditioning and washing steps are eliminated in the proposed method. The technique is called solvent extraction - molecularly imprinted polymer solid phase extraction (SE-MIP-SPE).

An interesting approach was used by Jarmalaviciene et al. (35). The authors demonstrated efficacy of restricted-access media solid phase microextraction method (RAM-SPME), compared to conventional fillings of monolithic columns C_{18} and reversed phase (RP) in simultaneous separation and determination of protein, caffeine, paracetamol and acetylsalicylic acid in biological material.

A similar analysis of the fillings was made by Chen et al. (36) in the determination of trace amounts of caffeine in sewage effluents. The Polymeric RP pre-column (PLRP-s column containing rigid macroporous spherical particles of polystyrene/divinylbenzene) has proved to have higher recovery efficiency and were more repeatable (repeatability, CV% 2.6) than other adsorbents compared: C_{18}, polymer RP (PRP-1 with a pore size 100Å) and Env (high purity styrene-divinylbenzene - SDVB) - CV%, respectively, 3.1, 3.7, 3.9.

CHROMATOGRAPHIC METHODS

High performance liquid chromatography (HPLC) (32, 33, 38–80, 183–185,188–191) and ultra-high performance liquid chromatography (UHPLC) (81–84) are particularly important in analyzing this group of compounds (Table 1).

Very interesting methods were developed for multicomponent systems. Di Pietro et al. (40) separated and quantified thirteen unmodified and modified purines in human urine in a single run. The method may be useful in the investigation of urinary pattern of methylated bases in diseases involving purine metabolism.

Zydroń et al. (64) developed a new gradient procedure that was successfully applied to the analysis of eleven methylxanthines and methyluric acids in urine of patients with chronic asthma treated with theophylline.

Georga et al. (73) reported automated RP-HPLC method, using a linear gradient elution, for simultaneous analysis of caffeine, theophylline and their ten metabolites.

Schneider et al. (76) developed an HPLC–ES-MS/MS method for the determination of eleven caffeine metabolites and two internal standards after a simple sample filtration.

A similar procedure without extraction was optimized for the determination of 1-methylxanthine (1-MX), 1,7-dimethyluric acid (1,7-DMUA), 1-methyluric acid (1-MUA) and 2 uracils as shown by Nyéki et al. (77). Initially the urine samples were diluted, centrifuged and then injected onto a YMC-Pack Polyamine II (250 × 4.6 mm) column applying an acetonitrile stepwise gradient elution program.

Sixteen purine derivatives (UA, 2,8-dihydroxyadenine, xanthine, hypoxanthine, allopurinol, oxypurinol and 10 methyl derivatives of UA or xanthine) in urinary caliculi were simultaneously determined by Safranow et al. (50). The limits of detection (LOD) for individual compounds ranged from 6 to 35 μg purine/g of the stone weight and precision (CV%) was 0.5–2.4%.

The largest number of compounds was analyzed in a gradient HPLC-UV/MS/MS method developed by Yang et al. (61). The authors determined 21 metabolites related to the diabetic nephropathy in human plasma with very good linear range (LR) and LOD values.

In all publication including HPLC methods MS or UV detectors were used. In only three articles, electrochemical detection was used when xanthines are electroactive. Inoue et al. (59) determined UA in human saliva, Yang et al. (62) reported UA analysis beside creatinine, cystine, oxalic acid and citric acid in urine samples and Li et al. (81) determined ascorbic acid (AA), dehydroascorbic acid and UA using homogentisic acid (HGA) as internal standard.

Gas chromatography (GC) is rarely used with xanthine (17,24,15). Obviously GC is exclusively dedicated to volatile compounds. Xanthines

TABLE 1 LC methods Used in Analysis of Methylxanthines

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine/pyridostigmine bromide, acetaminophen, acetylsalicylic acid/rat plasma and urine	HPLC	RP C18 column μBondapak C18 125 A 10 μm, 3.9 × 300 mm + guard column Supelco, 2 cm × 4.0 mm, 5 μm; gradient of 1–85% acetonitrile in water (pH 3.0) at a flow rate ranging between 1 and 1.5 mL/min; 280 nm	100–1000 ng/mL	100 ng/mL	–	[38]
Caffeine/8-chlorotheophylline, diphenhydramine/-	HPLC DAD.	SymmetryShield RP8 (25 × 0,46 cm); acetonitrile–(0.01 M H_3PO_4–triethylamine, pH 2.8) (22:78, v/v); 229 nm; 1 mL/min	0.125–0.376 mg/mL caffeine 0.063–0.188 mg/mL 8-chlorotheophylline	–	<2 0.7–1.3	[39]
Caffeine/catechins, gallic acid/different tea samples	HPLC	Spherisorb column (C18 RP) S10 ODS2; gradient A (water−acetic acid, 97:3 v/v) to solvent B (methanol), 1 mL/min; 280 nm	0.6−150.0 μg/mL	4.5 ng/mL	–	[10]
Hypoxanthine, xanthine, 1-MHX, 1-MX, 3-MX, 7-MX, 1,3-DMX, 1,7-DMX, 3,7-DMX, 1,3,7-TMX/urine	HPLC	Supelcosil LC-18 column (Supelco, 250 × 4.6 mm I.D. 5 μm) + precolumn (20 × 4.6 mm I.D.) with the same material; gradient A: 0.01 M potassium phosphate buffer (pH 5.5 with 0.5 M potassium hydroxide), B: methanol; 1.5 mL/min	0.1–10 nM	0.02 nM	0.21–2.44	[40]
1-MX, 1-MUA/-/-	HPLC (UV)	Hypersil ODS (5 μm particle size, 250 × 4.6 mm I.D.) + guard; 10 mM sodium acetate and 5 mM TBA, adjusted to pH 5.4 with NaOH; 1 mL/min; 273 nm	1–50 μg/mL	1.0 μg/mL	<15 <15 (n = 6)	[4]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine, 1-MX, 1-MUA/-/urine samles	LC-ES-MS	MIC 15 CP, C18, 3.5 μm column (150 × 1 mm I.D.; (A) 2 mM ammonium formate, pH 3 and (B) acetonitrile in gradient; 50 μl/min	0.25–50 μg/mL	0.25 μg/mL	4.7–9.8 6.0–17.25 (n = 6)	[4]
Caffeine, theobromine, theophylline/-/-	HPLC (UV)	LiChrospher 100 RP-18 (244 mm × 4.4 mm i.d., 5 μm) + guard LiChrospher column of similar characteristic (4 mm × 4 mm i.d.; Merck); 273 nm; water/ethanol/acetic acid (75:24:1%, v/v/v) as mobile phase; 1.0 mL/min	1.0–60 μg/mL (n = 10)	0.10 μg/L for caffeine, 0.07 μg/L for theobromine and 0.06 μg/L for theophylline	0.01–0.1	[41]
Caffeine/ergotamine), propyphenazone, camylofin, mecloxamine/tablets	HPLC	300 × 3.9 mm, 10 μm particle size) packed with μ-Bondapak C18; methanol-water-triethylamine (60+40+0.1, v/v/v); 1.5 mL/min, 254 nm	–	1.5 μg/mL	<5 (n = 5)	[42]
Caffeine, theophylline, theobromine/-/human plasma	SPE HPLC	short protein-coated μBondapak CN silica pre-column (20 × 3 mm, i.d.); 5 μm TSK gel ODS-80 TM, (150 × 4.6 mm i.d.); methanol-0.01 M phosphate buffer, pH 3.5 (30:70, v/v); 275 nm	0.5–20 μg/mL	2.22×10^{-2}; 2.41×10^{-2}; 2.17×10^{-2} μg/L	<3 <4	[43]
Caffeine/ Phenylpropanolamine HCl, Diazepam/tablets	HPLC (UV)	Supelcosil LC-18 (5 μm) gradient with acetonitrile:water (30:70 v/v); 0.4 mL/min; 254 nm	0.036–0.084 mg/mL	4.1 μg/mL	0.91	[44]

Theophylline, Etofylline/ Guaiphenesine, Ambroxol HCl/liquid dosage form	HPLC (UV)	RP Hypersil Phenyl BDS (25 cm × 4.6 mm, 5 mm); triethylamine pH 3.0 buffer: methanol (85:15) v/v; 1.5 mL/min; 235 nm	5–37 μg/mL for Theophylline, 19–140 μg/mL for Etofylline	–	<2	[45]
UA/creatinine/urine	HPLC (UV)	Supelcosil RP C-18 (25 cm × 4.6 mm id. 5 μm)+ RP-18 guard column; 0.05 M ammonium phosphate buffer at pH 7.4; 235 nm	0.25–25 μg/mL	0.127 μg/mL	0.98	[46]
UA/AA, dehydroascorbic acid/human plasma	HPLC-ECD	YMC ODS-AQ (3.0 mm × 150 mm, 3.0 μm+4 mm × 20 mm, 5 μm guard (A) methanol; (B) 150 mM chloroacetic acid, 2 mM disodium EDTA, pH 3.0 with 10 N sodium hydroxide; 300 μl/min	–	0.2 μM	0–7.2 2.1 (n = 4)	[81]
UA/AA/human plasma	UHPLC	Hypersil Gold C18 columns (one 2.1 mm × 100 mm, one 2.1 mm × 20 mm, both 1.9 μm + one OPTI-GUARD EXP guard column 2.1mm × 5mm; (A) methanol; (B) 150 mM chloroacetic acid, 2 mM disodium EDTA, pH 3.0 with 10 N sodium hydroxide; 600 μl/min	–	0.3 μM	0.4–3.7 5.4 (n = 4)	[81]
Caffeine/sewage	HPLC (DAD)	Hypersil C18 (250 mm × 4 mm i.d. × 5 μm); 1 mL/min, gradient of water-acetonitrile; 210 nm	0.1–10 μg/L	0.1 μg/L	3.1	[58]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine, theobromine/catechins, gallic acid, strictinin/green tea	HPLC (UV)	Wakopack Navi C18-5 columns (5 μm, 4.6 mm 150 mm) + guard column (5 μm, 4.6 mm × 10 mm); (A) comprised 0.25% v/v phosphoric acid and acetonitrile (20:1 v/v, pH 2.4), (B) comprised mobile phase A and methanol (5:1 v/v, pH 2.5); 1.0 mL/min	0.61–46.48 μg/mL, 1.13–67.92 μg/mL	0.24 μg/mL, 0.44 μg/mL	0.4–2.9, 3.8–8.4 0.6–3.4, 3.5–8.3	[11]
Caffeine/chlorzoxazone, tolbutamide, metoprolol, midazolam/rat plasma	HPLC (UV/DAD)	C(18) column (5 μm particle size, 250 × 4.6 mm i.d.); methanol and 50 mM phosphate buffer (pH 3.4, 65:35) 1 mL/min; 230 nm	0.2–50 μg/mL	0.2 μg/mL	1.38–11.10 3.39–11.33	[47]
UA/creatinine/human urine	HPLC	C18 RP (150 × 3,00i.d. 5 μm;Waters) + 10 mm C18 guard; gradient (A) sodium phosphate buffer; pH 4.75, (B) acetonitrile, 250 nm	0.00–25.00 μg/mL	0.062 μg/mL	0.32–0.65	[48]
Caffeine/acetaminophen, butalbital/human serum	HPLC	Chromolith performance RP-18e (10 cm × 4.6 mm ID) + guard C18 (7.5 mm × 4.6 mm); 95:5 (v/v) 0.1 M potassium phosphate monobasic (pH 2.41)–acetonitrile; 9 mL/min	1.25–100 μg/mL	1.25 μg/mL	<8.3	[49]

UA, 2,8-dihydroxyadenine, xanthine, hypoxanthine, allopurinol, oxypurinol, 1-MUA, 3-MUA, 7-MUA, 9-MUA, 1,3-DMUA, 1,7-DMUA, 3,7-DMUA, 1-MX, 3-MX, 7-MX/urinary calculi	HPLC	mm × 4 mm column pre-packed with LiChrospher 100 RP-18 (5 μm particles, 0.08 nm pores) (Merck) + cartridge 4 mm × 4 mm, packed with the same material, served as precolumn; (A) 50 mM KH_2PO_4 (pH 4,6), (B) 50 mM KH_2PO_4/K_2HPO_4 (pH 6,4), (C) methanol	0–100 mg/L; (25 mg/L for Xanthine)	0.006–0.035 mg purine/g of the stone weight	0.5–2.4	[50]
Caffeine/acetaminophen, atorvastatin, carbamazepine, ciprofloxacin, fluoxetine, levofloxacin, sertraline, sulfamethoxazole, trimethoprim /surface water	LC–MS/MS	UPLC 50.0 × 2.1mm, bridged-ethyl-siloxane/silica hybrid (BEH) Shield RP-C18 column + 30.0 mm C18 guard column; gradient of 95:5 MQ:ACN+0.1% FA as the aqueous phase (A) and ACN+0.1% FA as the organic phase (B).	–	0.990 pg	2.9	[9]
UA/serum	HPLC/ID-MS	Inertsil ODS-SP (150 mm × 2.1 mm i.d.), 5 μm; 100% ammonium acetate 10 mM at pH 4.5; 0.2 mL/min	6.0–200 ng/kg	0.032 ng	0.10–0.15	[51]
Caffeine/paracetamol, amantadine HCl, chlorpheniramine maleate/human plasma	HPLC-ESI-MS/MS	commercially available column (150 mm × 2.1 mm I.D., 5 μm)	20–2000 ng/mL	20 ng/mL	<15	[52]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine/cocaine, amphetamine-related compounds, LSD, ketamine, PCP, fentanyl and metabolites, nicotine and their metabolites/drugs in surface and waste waters	UPLC–MS/MS	Acquity BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm particle size); (A) acetonitrile with 0.1% formic acid, (B) 30 mM formic acid/ammonium formate (pH 3.5); 0.5 mL/min.	40–5500 ng/L	Surface water 1.5 ng/L Wastewater 5.0 ng/L	3.8 5.0 5.6 7.1	[82]
Caffeine/paracetamol, pseudoephedrine, chlorpheniramine, cloperastine/human plasma	LC–MS/MS	Venusil Mp-C(18) (50 mm × 4.6 mm, 5 μm); formic acid:10mM ammonium acetate:methanol (1:40:60, v/v/v)	10–4000 ng/mL	–	3.3–5.9 2.7–6.9	[53]
Caffeine, theobromine, theophylline/dietary supplements	LC-MS	Phenomenex Luna C18 (150 mm × 4.6 mm, 3 μm); water/methanol/acetic acid (75:20:5, v/v/v); 0.7 mL/min	0.06–500 μg/mL for caffeine, respectively; 0.06–100 μg/mL for theobromine and theophylline	0.2–0.3 μg/mL	3.5–11.5 (n = 5) 3.4–10.5 (n = 15)	[54]
Caffeine/carbamazepine, atenolol, griseofulvin, acycloguanisine, propranolol, ampicillin, verapamil/drugs	UPLC/MS/MS	Acquity UPLC BEH C18 (50 mm × 2.1 mm, i.d., 1.7 μm particle size); 55° C; 0.1% formic acid in water (A), 0.1% formic acid in acetonitrile (B)	12.21–6250 nM	3.05 nM	3.03–12.32	[83]

Theophylline anhydrous/guaiphenesin, diphenhydramine HCl, methylparaben, propylparaben, sodium benzoate/pharmaceutical syrup	HPLC (UV)+ PLS-1/PCR	Shim-pack RP18 (250 mm × 4.6 mm i.d.) (4.6 μm particle size); 25 mM KH_2PO_4, pH 3.2—acetonitrile (60:40, v/v); 222 nm	5.0–33.0 μg/mL	1.59×10^{-2} μg/mL	0.80	[56]
Theobromine, caffeine/ syringic acid, naringenin, myricetin, kaempferol, ferulic acid, *o*-coumaric acid, *p*-coumaric acid, cinamic acid, quercetin, apigenin, genistein, luteolin, gallic acid/carob flour	UPLC-MS/MS	Acquity High Strength Silica (HSS) (100 mm × 2.1 mm i.d., 1.8 μm T3); (A) water/acetic acid (0.2%), (B) acetonitrile; 0.4 mL/min	0.05−20 μg/mL; 0.002−20 μg/mL	0.03 μg/mL; 0.001 μg/mL	0.9; 1.9 (n = 3)	[23]
UA/-/urine, saliva	LC/MS/MS	Zorbax Sax Column (150 mm × 4.6 mm i.d.), an anion exchange resin, 5 μm particle size with a 12.5 mm × 4.6 mm i.d. guard column; 50% sodium citrate 1 mM at pH 6.5, 50% acetonitrile; 1 mL/min	0.07–10 mg/l	0.21 ng	–	[57]
Caffeine/nicotine, cotinine, trans-3-hydroxycotinine, cotinine-N-oxide, arecoline/breast milk	LC/MS/MS	Eclipse XDB-C8 column (100 × 3.0 mm, 3.5 μm) a gradient of (A) 50 mM ammonium formate, pH 5.0, (B) acetonitrile; 0.5 mL/min	6–850 μg/L	1,6 μg/L	3.5–7.8 (n = 5) 3.9–14.2 (n = 15)	[58]
Theobromine, Caffeine/saliva, plasma, urine	UPLC–MS/MS	C18 bridged-ethyl hybrid (BEH) column 1.7 μm particles × 2.1 mm × 50 mm; (A) 0.1% (v/v) formic acid in water, (B) acetonitrile; 0.6 mL/min	2.5–400 μM/L	1.25 μM/L	3.0–14.6 (n = 10) 5.1–17.0 (n = 25)	[84]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
UA/-/human saliva	HPLC (UV)	CAPCELL PAK C18 UG 120 (150 × 2.0 mm, 5 μm); potassium phosphate buffer (74 mM, pH 3.0); 0.2 mL/min; 284 nm	0.6–60 μM	180 nM	2.2	[59]
UA/-/human saliva	HPLC (ED-Amp)	CAPCELL PAK C18 UG 120 (150 × 2.0 mm, 5 μm); potassium phosphate buffer (74mM, pH 3.0); 0.2 mL/min; GCE; E=+600mV vs. Ag/AgCl	0.06–6 μM	3 nM	0.8	[59]
Theophylline, 1-MX, 3-MX, 1-MUA, 1,3-DMU/plasma	LC-MS/MS	YMC Hydrosphere C18 column (50 × 2.0 (i.d.) mm; 3 μm bead size) 70:30 (v/v) methanol and aqueous 0.5 mL/L acetic acid; 0.25 mL/min;	0.625–20 mg/L for theophylline and 0.063–2 mg/L for metabolites	0.0280 mg/L for theophylline and 0.0059–0,0234 mg/L for metabolites	1,6 (n = 7) for theophylline; 1,6–7,8 (n = 7) for metabolites 7,4 (n = 6) for theophylline; 2,7–9,9 (n = 6) for metabolites	[60]
Caffeine/-/food samples	HPLC	RP-18e monolithic column Chromolith SpeedRod (50 mm × 4.6 mm i.d.); ACN/water (10:90, v/v); 3.0 mL/min	0–200 mg/L	0.10 mg/L	0,08–0,47 (n = 6)	[12]

UA/creatine, creatinine, orotic acid, β-alanine, cytosine, uracil, dihydrouracil, cytidine, hypoxanthine, uridine, xanthine, thymine, deoxyuridine, inosine, guanosine, deoxyinosine, thymidine, adenine, adenosine, deoxyadenosine/plasma	HPLC–UV/MS/MS	Agilent TC–C18 (250 mm × 4.6 mm I.D., 5μm particle size) + Alltech guard column (7.5 mm × 4.6 mm I.D., 5μm particle size); (A) 10mM ammonium acetate in Ultra pure water adjusted to pH 5.8 with glacial acetic acid, (B) 100% methanol; 0.8 mL/min	2–200 μg/mL, 0.02–2 μg/mL, 0.2–20 μg/mL	0.05 μg/mL, 0.02 μg/mL, 0.1 μg/mL	3.12–5.25; 2.38–4.37; 3.33–5.57 (n = 5) 5.16; 6.68; 9.01 (n = 6)	[61]
UA/creatinine, cystine, oxalic acid, citric acid/urine	HPLC-(ED-CV)	Hamilton RCX-10 anion-exchange column (250mm × 4.1mm i.d.); 0.1M phosphate buffer; Cu″SPE vs. Ag/AgCl	0,05–100 μM	10 nM	5.3	[62]
Caffeine, theophylline/ acetaminophen, phenytoin, ranitidine /plasma	LC-MS/MS	Luna 3μm C18(2) column (150 mm × 3 mm); gradient; (A) water and methanol (95:5, v/v), (B) water and methanol (10:90, v/v), both containing 0.05% formic acid; 0.3 mL/min;	48.8–25000 ng/mL, 24.4–25000 ng/mL	48.8 ng/mL, 24.4 ng/mL	6.0–8.8 6.6–13.5	[63]
Caffeine/paraxanthine/ human plasma and urine	LC-MS/MS	Waters symmetry C18 guard cartridge (3.9 mm × 20 mm, 3.5 μm); acetonitrile and 0.1% formic acid in water (70:30); 0.3 mL/min.	0.05–5 μg/mL	1–100 ng/mL	6.4–13.7 6.4–14.3	[5]
Caffeine, theobromine, theophylline, paraxanthine, 1-MX, 3-MX, 7-MX, 1-MUA, 1,3-DMUA, 1,7-DMUA, 1,3,7-TMUA/urine	HPLC	LiChrosorb RP-18 (7μm) 100-4,6 column; gradient, (A) 0.05% aq. solution of trifluoroacetic acid, (B) acetonitrile; methylxanthines at 274 nm, methyluric acids at 289 nm	0.100–50000 μg/mL	0.050–0.070 μg/mL	1.1–2.6 (n = 3) 2.0–4.9 (n = 3)	[64]
Caffeine/catechins/tea	SPME-HPLC-ESI-MS/MS	Supelcosil LC-18 (150 mm × 4.6 mm i.d. × 5 μm); 0.3 mL/min	0.5–500 ng/mL	0.01 ng/mL	1.3–5.5 4.5–8.6	[13]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Theophylline/ephedrine HCl/tablets	HPLC	Bondopak C18 RP packed with 10 μm dimethyl octadecylsilyl bonded amorphous silica (300 × 3.9 mm); methanol+water (40+60, v/v) adjusted pH 3 with 10% v/v *o*-phosphoric acid; 1.2 mL/min; 217 nm	20–180 μg/mL	0.59 μg/mL	<1.5	[65]
Theophylline/-/human serum samples	SE-MISPE, HPLC(UV)	Spherisorb ODS, 5μm, 4.6mm i.d. × 22 cm; 15 mM potassium phosphate (pH 4.9)/methanol (85/15, v/v); 1.75 mL/min, 274 nm	0.5–30 μg/mL	0.9 μg/mL,	3.5 (n = 3)	[35]
UA/-/urine, plasma, cell lysate	LC-MS/MS	Phenomenex Luna C18(2) column (150 × 4. 6mm, 5 μm)+guard Phenomenex Luna C18(2), 5 μm; (A) 5 mM ammonium acetate/0.1% acetic acid, (B) methanol; 0.6 mL/min	1.00–16.00 mg/dl (urine,plasma), 8.40 μg/dl-4.00 mg/dl (cell lysate)	0.84 μg/dl	0.01–3.07 (n = 5) 0.01–3.68 (n = 5)	[66]
1-UA; 1-MX; 1,7-DMX caffeine/-/-	HPLC ES-MS/MS	YMC-Pack C30; Gradient (A) 0,5% acetic acid, (B): 100% acetonitrile, 200 μl/min	2 nM-50 μM	–	0.2–18.3 0.1–22	[67]
Hypoxanthine/-/rat brain	HPLC	HP ODS Hypersil 4.6 mm i.d. × 200 mm, 5 μm; Nafion-GOD/XOD-$PdIrO_2$/GCE 0,70V vs. Ag/AgCl/amp.; phosphate buffer of pH 6.9	1.0×10^{-6}–5.0×10^{-4} M/l	2.0×10^{-7} M/l	4.2 (n = 11)	[68]

Xanthine, hypoxanthine/-/-	LC	SepStik 3 μm, C 18, silica microbore column, 100 mm × 1 mm i.d; XOD/$CuPtCl_6$/GC; 0.025 M phosphate buffer solution (pH 7.4)	6×10^{-7}–2×10^{-4} M, 5×10^{-7}–2×10^{-4} M	1×10^{-7} M	2.7, 3.0	[69]
Caffeine/sulfacetamide	HPLC	RP Gemini C18 (150 mm × 4.6 mm, 5 μm) coupled with a C18 guard; A: 50 mM potassium dihydrogen phosphate in water (pH 5.5 adjusted with 1M NaOH), B: ACN. 1.0 mL/min.; 273 nm	0.04–4.50 mh/L	0.01 mg/L	–	[118]
Caffeine, theophylline, paraxanthine, theobromine/-/serum	SPE HPLC	Oasis HLB 30 mg/1cc; Shimpack CLC-ODS 6 mm × 15 cm; 0.1M NaH_2PO_4 in 30% methanol; 274 nm; 1 mL/min	1.0–100 μg/mL (caffeine), 0.2–20 μg/mL (others)	1.0 μg/mL (caffeine). 0.2 μg/mL (others)	CV%: 1.15–1.84 (caffeine), 0.77–4.78 (others)	[70]
UA/-/plasma	HPLC	Luna C18 (3 μm; 150 × 4.6 mm i.d.) + SecurityGuard C18 guard column; 0.60 mL/min; one liter of mobile phase consisted of 17.8 g sodium dihydrogen phosphate monohydrate (129 mM), 17.6 g sodium acetate anhydrous (215 mM), 372 mg disodium-EDTA(1.0 mM), 50 mg n-dodecyltrimethylammonium chloride (189 μM) in 750 mL H_2O mixed with 20mg tetraoctylammonium bromide (36.6 μM) dissolved in 250 mL methanol, pH 5.4	25–500 μM	–	<1	[71]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine/-/-	HPLC	Ultrasphere ODS column with 3 μm particle size (7.5 cm × 0.46 cm i.d.); 280 nm; water/acetonitrile (90:10, v/v); 1 mL/min	6–30 μg/mL	0.7 μg/mL	–	[72]
7-MUA, 1-MUA, 7-MX, 3-MX, 1-MX, 1,3-DMUA, theobromine, 1,7-DMUA, paraxanthine, theophylline/-/blood serum, urine	SPE HPLC (UV)	Nexus SPE, MZ Kromasil C4, 250 × 4 mm, 5 μm; acetate buffer (pH 3.5)-methanol (97:3, v/v) changing to 80:20 v/v in 20 min time, 1 mL/min; 275 nm	up to 8 ng/μl except paraxanthine and theophylline, for which is up to 12 ng/μl; caffeine for which is up to 20 ng/μl.	0.3 ng/10 μl injection		[73]
Theophylline/etofylline/ human plasma	HPLC UV	Intertsil C18 (5μm, 150 mm × 4.6 mm i.d); 10mM ammonium acetate buffer/methanol/acetonitrile (86:7:7, v/v/v); 1.0 mL/min; 272 nm	100–10,000 ng/mL	100 ng/mL	<6.0	[74]
Theophylline, theobromine, caffeine, paraxanthine/-/urine	HPLC DAD	5μm Supelcosil LC-18-DB (250 × 2.1 mm i.d.) + 5 μm Supelguard LC-18-DB (20 × 2.1 mm i.d.); 20:80 methanol:buffer (5mM citric acid adjusted to pH 5 with triethylamine); 0.2 mL/min; 224–380 nm	2 ng/mL – 2 μg/mL	0.66 ng/mL - 0.8 ng/mL	–	[75]

caffeine, theobromine, theophylline, paraxanthine, 1-MX, 3-MX, 7-MX, 1-MUA, 3-MUA, 7-MUA, 1,3-DMUA, 3,7-DMUA, 1,7-DMUA, 1,3,7-TMUA/ 5-acetylamino-6-amino-3-methyluracil, 5-acetyl-amino-6-formylamino-3-methyluracil, /urine	HPLC MS/MS	Ultrasphere ODS column (250 × 4.6 mm, 5μm; a gradient of 0.05% acetic acid (v/v) with 3% methanol (v/v) and 1.5% of 2-propanol (v/v) (solvent A), methanol (solvent B), and 0.05% acetic acid (v/v) (solvent C);	1-MUA 0.25–15 μM, 1,3-MUA 0.125-7.5 μM, 1,3,7-TMUA 0.05–12.5 μM, all other compounds 0.05–25 μM	0.05 μM (1 pM/injection) for most metabolites, 0.1 μM (2 pM/injection) for 1,3-MUA	1.3 - 10.3	[76]
1-MX, 1,7-DMUA, 1-MUA/5-acetylamino-6-formylamino-3-methyluracil, 5-acetylamino-6-amino-3-methyluracil/urine	HPLC UV	YMC-Pack Polyamine II (250 × 4.6 mm); acetonitrile-0.75% (v/v) formic acid: (91:9) in gradient; 1.0 mL/min; at 260 nm for 1-MX and AAMU, and at 280nm for AFMU, (1,7-DMUA) and (1-MUA)	15–120 μM	–	<4.2 <9.4	[77]
UA, hypoxanthine, xanthine/allantoin/-	HPLC PAD	Nova-Pak C18 columns (4μm, 250 × 4.6 mm, Waters) + guard column (Waters) of 10 × 6mm containing reversed-phase C18 (30–40μm) pellicular packing material; A: 0.0025 M $NH_4H_2PO_4$ buffered to pH 3.5 with 10% phosphoric acid, B: was solvent A–methanol (95:5, v/v); 284, 250 and 267 nm	151–1515 μM; 117–1174 μM; 69–696 μM	0.21 nM; 0.23 nM; 0.70 nM	–	[78]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
3-MX, 1-MX, theobromine, caffeine, 1,3-DMUA, 1,7-DMUA, paraxanthine, theophyllinum, 1,3,7-TMUA/-/-	HPLC UV	Chromolith Performance PR-18e 10 cmx4.6 cm; 10 mM potassium dihydrogen phosphate buffer/methanol (87.5:12.5 v/v), 1 mL/min.; 274 nm	0.03–40 μg/mL (3-MX, 1-MX, theobromine, caffeine); 0.05–40 μg/mL (1,3-dimethyluric acid, theobromine, 1,7-DMUA, paraxanthine, theophyllinum, 1,3,7-TMUA	0.0045 μg/mL 3-MX, 0.0051 μg/mL 1-MX, 0.0106 μg/mL theobromine, 0.0058 μg/mL caffeine, 0.0097 μg/mL 1,3-DMUA, 0.0107 μg/mL 1,7-DMUA, 0.0071 μg/mL paraxanthine, 0.0136 μg/mL theophyllinum, 0.0074 μg/mL 1,3,7-TMUA	0.21–1.86 / 0.23–2.55	[79]
Caffeine/acetylsalicylic acid, paracetamol, phenobarbital/tablets	HPLC UV	Bio SiL HL C18, 5 μm, 250 × 4.6 mm; MeCN–water (25:75 v/v) adjusted to pH 2.5 with H_3PO_3, 2.0 mL/min; 273 nm	0.01–0.08 mg/mL	1.7×10^{-4} mg/mL	0.36–1.52 / 0.58–2.18	[8]
Hypoxanthine/inosine/ human plasma	HPLC DAD	Phenomenex Onyx monolithic C18, 20 cm × 4.6 mm I.D., 130Å column coupled to an Onyx™ C18 guard column, 5 cm × 4.6 mm I.D.; 0.1% TFA in deionized water, pH 2.2, v/v) and methanol gradient; 250 nm	0.25–5 μg/mL	100 ng/mL	2.2–7.5	[80]

Pentoxifylline, LSF /-/plasma	LC-(ESI) MS/MS	Zorbax SB-C18, Rapid Resolution Cartridge (30 × 2.1 mm i.d.) and 3.5 μm particle size + Phenomenex Guard Cartridge C18, 4 × 2 mm; 35°C; methanol + aqueous 0.1% formic acid solution (1:3, v/v); isocratic; 0.8 mL/min.	1–500 ng/mL	1 ng/mL	–	[183]
Pentoxifylline, LSF /-/plasma	LC-(ESI) MS/MS	Zorbax SB-Aq, StableBond Analytical (150x 4.6 mm i.d.) and 5 μm particle size + Phenomenex guard cartridge; 35°C; methanol + aqueous 0.1% formic acid solution (3:2, v/v); isocratic; 0.8 mL/min.; 7.5 min.	1–500 ng/mL	1 ng/mL	–	[183]
Pentoxifylline, LSF /-/rat serum,tissues	HPLC UV	ChiralPak AD column (250 mm × 4.6 mm i.d., 5 mm); hexane/2-propanol (84:16, v/v) + 0.01% diethylamine; 1.5 mL/min	0.01–100 mg/mL	0.01 μg/mL	–	[184]
Pentoxifylline/-/plasma	HPLC UV	Altima C18 (250 × 4.6 mm id, 5μm, 250Å) + guard Altima C18 (7.5 × 4.6 mm id, 5 μm); methanol-acetonitrile-mixed phosphate buffer (pH 2.6; 10 mM; 39+12+49 v/v/v); 1.0 mL/min.; 273 nm; 10 min.	15–400 ng/mL	5 ng/mL	>3.8 >8.8	[185]

(*Continued*)

TABLE 1 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Pentoxifylline/-/human plasma	LC-MS-MS	XTerra MS C18 column (3.5 μm, 100 × 3.0 mm i.d.); 30°C; 10 mM ammonium formate buffer-acetonitrile (30:70, v/v); isocratic; 0.6 mL/min.	2–1000 ng/mL	2 ng/mL	>10	[188]
Pentoxifylline/-/human plasma	HPLC	Nucleosil-100 C18 RP Column (250 × 4 mm i.d.) 5 μm particle size; acetonitrile–water, 45:55 (v/v); 1 mL/min.	25–1000 ng/mL	20 ng/mL	–	[189]
Dyphylline/guaiphenesin, methylparaben, propylparaben/syrup	HPLC UV	Phenomenex Luna 250 × 4.6 mm (i.d.), 5 μm RP C_{18}; 20 mM KH_2PO_4 pH 3.3-acetonitrile (55+45, v/v); 1.5 mL/min; 260 nm; 10 min.	3–19 μg/mL	1.97×10^{-2}	0.81	[190]
Dyphylline/phenobarbitone, papaverine HCl/-	HPLC UV	HAISIL100 C_{18} 250 × 4.6 mm (i.d.), 5 μm ; 20 mM KH_2PO_4 pH 3.5-acetonitrile (55+45, v/v); 1.5 mL/min; 210 nm	10–25 μg/mL	2.66×10^{-4}	0.013	[191]

MHX, methylhypoxanthine; *MX,* methylxanthine; *DMX,* dimethylxanthine; *TMX,* trimethylxanthine; *UA,* uric acid; *AA,* ascorbic acid; *MUA,* methyluric acid; *DMUA,* dimethyluric acid; *TMUA,* trimethyluric acid; *LSF,* lisofylline.

are not naturally very volatile; they will require extensive sample preparation and derivatization to be analyzed by GC. However Gómez et al. (24) determined by GC at 220°C underivatized caffeine and 1,7-dimethylxanthine beside nicotine, ibuprofen, acetaminophen, DCDD (2,7-dichlorodibenzo-*p*-dioxin), Triclosan, Diclofenac, Bisphenol A and Carbamazepine in waste water with a GC ramp reaching 300°C. This new multi-residue analytical GC method based on solid phase extraction (SPE) with Oasis HLB sorbent gave LR from 10 to 1000 ng/L and 50 to 1000 ng/L for caffeine and 1,7-dimethylxanthine, with LOD of 0.7 and 10 ng/L respectively. The method was easy, fast and viable for routine analysis, avoiding the inconvenience associated with the application of derivatization processes. Two other GC works considered caffeine (17) and caffeine beside guarana (15). The LRs were 0.05–5.0 mg/L with LOD 4.0 μg/L and 50–1000 mg/L with LOD 15 mg/L respectively.

Thin-layer liquid chromatography (TLC) (85,187) was used in two works, and it was supplanted by the more modern variety: high performance TLC (HPTLC) (19,20,86,186) (Table 2). Formerly TLC was employed only to identification purposes, but combined with modern forms of detection (e.g., densitometry) (19,86) it is a great tool in analytical laboratory. The mobile phases were composed of two solvents: ethyl acetate–methanol (19,187), three solvents: ethyl acetate–methanol–ammonia (86) or acetic acid-isopropanol-toluene (85), four solvents: acetone-chloroform-toluene-dioxane (186) and even five solvents: chloroform- ethanol-acetic acid-acetone- water (20). The typical UV wavelength for detection of caffeine is 274–275 nm and for theophylline 272 nm.

Mirfazaelian et al. (85) used a TLC method for the determination of theophylline in plasma; the other works were regarding caffeine in pharmaceutical formulations and food products.

A very interesting procedure was reported by Aranda et al. (86); the authors coupled a HPTLC system to a ESI-MS detection method using stable isotope dilution analysis (SIDA). They could quantify very precisely caffeine in pharmaceutical and energy drink samples (samples and caffeine standard were over-spotted with caffeine-d_3).

ELECTROPHORESIS

The capillary electrophoretic methods (CE) (7, 14, 16, 18, 21, 22, 35, 87–115,116–118, 192) were also widely used in the analysis of methylxanthines. Depending on the use of electrolytes and buffers, on the types of columns and on the separation mechanism, two CE methods can be distinguished: the capillary zone electrophoresis (CZE) and the micellar electrokinetic capillary chromatography (MEKC) methods (7, 14, 16, 22, 87, 89, 91, 99, 116, 118).

TABLE 2 HPTLC and TLC Methods Used in Analysis of Xanthines

Compound/simultaneously determinate with/sample matrix	Separation method	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Theophylline/-/plasma	TLC UV	SG60 F_{254}, 10 × 20 cm, 0.25 mm layer thickness, Merck; acetic acid, isopropanol, and toluene (1: 12: 6); UV 272 nm	500–10,000 ng/mL	100 ng/mL	4.4 7.8	[85]
Caffeine/-/herbal products, power drinks	HPTLC UV	SG60 F_{254} Merck (20 × 10 cm); ethyl acetate–methanol (85:15, v/v); densitometry; UV 275 nm	214.00–1284.00 ng/spot	–	<5	[19]
Caffeine/pharmaceutical, energy drink	HPTLC UV/ESI-MS/ SIDA	SG60 F_{254} HPTLC; chloroform, ethanol, acetic acid, acetone and water (54:27:10:2:2, v/v/v/v) for energy drinks;ethyl acetate, methanol and ammonia (25%) (90:15:1, v/v/v) for pharmaceutical tablets;); densitometry; UV 274 nm	50–500 ng/band	0,75 ng/band	3,75; 1,44 (n = 6)	[86]
Caffeine/riboflavin, pyridoxine, nicotinamide, taurine/energy drinks, beverages	HPTLC UV/ESI-MS	SG60 F_{254} HPTLC Merck, 20 cm × 10 cm, 200 μm in thickness; chloroform, ethanol, acetic acid, acetone, water 54:27:10:2:2 (v/v); UV 275 nm	201.8–1009.0 ng/band	22 ng/band	4.4, 2.5 and 4.4 (n = 5)	[20]
Pentoxifylline/impurities/ side reaction products	HPTLC	LiChrospher® RP-18 F254s chromatoplates, acetone-chloroform-toluene-dioxane (2:2:1:1 v/v); 275 nm	400–600 ng/spot	0.1–0.6%	–	[186]
Pentoxifylline/mebendazol/FLC		20 × 10 cm glass-backed silicagel 60 F_{254} HPTLC plates (Merck); ethylacetate–methanol(85+15,v/v;7); a subsequent densitometric or video densitometric quantitation	0.9–1.4 μg	–	3.4	[187]

The ionizable uric acid (UA) is the compound most commonly analyzed using electrophoretic methods (94, 95, 103, 107, 110). UA is analyzed with ascorbic acid (AA) (90, 92, 104, 105, 108, 109, 111). All analyses were based on biological samples.

Georgakopoulos et al. (111) examined the levels of UA, AA and malondialdehyde in tears and obtained a LR of 2–500 μM and LODs of 0.5 μM.

An interesting method based on gold nanoparticle-enhanced CE-chemiluminescence (CL) detection was developed by Zhao et al. (107,108). In this way, traces of UA were determined in human serum and urine; the results were comparable with those obtained using UV detection. H_2O_2 (107) or $K_3(Fe(CN)_6)$ (108) were needed as post-column oxidizing agents to induce chemiluminescence.

There was only one paper concerning the simultaneous determination of UA and dopamine by CE (96). UA was also determined with creatinine and creatine in the works of Clark et al. (88), Garcia et al. (98) and Lee et al. (100).

Hypoxanthine (112) and hypoxanthine associated to UA and xanthine were also determined by CE (97, 117).

Caffeine, theophylline and theobromine (16, 18, 91, 93) were the object of CE analysis in food samples. Only Zhang et al. (106) reported the levels of caffeine and theophylline in rat serum and urine.

Caffeine beside paracetamol was also analyzed by CE by Kölhed et al. (99) simultaneously with p-nitro benzyl alcohol, m-nitrophenol and p-nitrophenol. The authors used infrared spectrophotometry with Fourier transformation (FTIR) as the detection method. Although the LR and LOD were in the mM range, it may be possible to reduce them by exploiting the stacking and sweeping possibilities provided by CE and MEKC or more powerful light sources. The two advantages of this method are that it does not destroy the analytes and the flow cell allowed for real-time identification results. MEKC coupled with FTIR might be of great interest of wide range of applications due to the provided unique IR spectrum of each separated analyte.

The electrophoretic methods also include capillary electrochromatography (CEC), which combines the high efficiency of CE with the high selectivity of LC. The CEC method was used by Ohyama et al. (101) to separate caffeine and its two metabolites: 1-methylxanthine (1-MX) and 1,7-dimethylxanthine (1,7-DMX). The stationary phase was a 3-(1,8-naphthalimido) propyl-modified silica gel (NAIP) and the optimal separations conditions were achieved with a 4.0 mM citrate buffer (pH 5.0) containing 80% methanol at an applied voltage of 25 kV. The compounds were completely separated with good repeatability in less than 3.5 minutes, which was approximately 3-times faster than HPLC with the same phase.

Microchip electrophoresis has become a mature separation technique in recent years. Microfluidic channels provide a stable electroosmotic flow

(EOF) because of the neutral hydrophilic surface chemistry, automation, fast analysis speed and minimum sample requirement. The most frequently used material was poly-dimethylsiloxane (PDMS) (90, 96, 98, 105, 106) (Table 3).

ENZYMATIC BIOSENSORS

Methylxanthines biosensors consist of a thin layer of the specific oxidase enzyme immobilized on the various types of electrode's surface, and an electron mediator Me (Equation (1)) can be oxydized. As a result of the reaction, the stoichiometric amount of dihydrogen dioxide and Me + occurred, whose redox potential is measured (Equation (2)).

$$H_2O_2 + 2Me + 2H^+ \longrightarrow 2H_2O + 2Me^+ \quad (1)$$

$$Me^+ + e^- \longrightarrow Me \quad (2)$$

Biosensors with Urate Oxidase (UOx)

Uricase catalyzes the oxidation of UA to allantoin as presented by Equation (3a):

$$\text{Uric acid} + O_2 \xrightarrow{\text{Urate oxidase (UOx)}} \text{Allantoin} + H_2O_2 \quad (3a)$$

UA was determined alone (119–124). Akyilmaz et al. (119) and Zhao et al. (123) analyzed it in urine, Zhang et al. (121) in blood samples and Zhang et al. (122) in rat stratium.

Biosensors work generally in phosphate buffer in a pH range 6.8 to 7.5 except in the Wang et al. work (120) where pH 8.5 was used. The LR and LOD covered millimole (mM) and micromole (μM) concentrations.

Akyilmaz et al. (119) pointed out, that the produced H_2O_2 undergoes a non-enzymatic decomposition releasing oxygen, which contributes to the total concentration of dissolved oxygen (DO) at the electrode surface, therefore, resulting in ΔDO values that are much lower than expected. To eliminate this effect he proposed a dual enzymatic system sensor (urikaza-peroxidase), working according to the mechanism described by Equations (3b) and (4):

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Urate oxidase (UOx)}} \text{Allantoin} + CO_2 + H_2O_2 \quad (3b)$$

$$\text{Uric acid} + 2H_2O_2 \xrightarrow{\text{Peroxidase (HRP)}} \text{Oxide donor} + 2H_2O \quad (4)$$

The significantly low LOD (5.0×10^{-8} mol/L) from microdialysis sampling was obtained by Zhang et al. (122) with a novel reagentless amperometric

TABLE 3 CE Methods Used in Analysis of Xanthines

Compound/simultaneously determinate with/sample matrix	Separation metod	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Caffeine/catechins, gallic acid, theanine, AA /dried fresh tea leaf, black tea, bottled black tea	MEKC	Tea leaf and black tea 100mM SDS, 25mM phosphate, 6% (v/v) methanol pH 7.0; bottled black tea 5% (v/v) methanol	5–75 μg/mL	2.0 μg/mL	1.18–4.07	[14]
Caffeine/acetaminophen, salicylamide, phenylephrine, pseudoephedrine, norephedrine, chlorpheniramine/ pharmaceutical preparations	MEKC	tris-borate (20 mM, pH 8.5); 274 nm	5–30 μg/mL	1.0 μg/mL	1.17 (n = 5) 4.82 (n = 5)	[87]
UA/creatinine, creatine, p-aminohippuric acid /urine	CE	Three run buffers: pH 9.5 borate buffer, pH 7.5 phosphate buffer, pH 5.5 phosphate buffer; 214 nm	1.5–250 μM	15 μM	10.9	[88]
Caffeine/paracetamol, propyphenazone/ pharmaceutical preparations	MEKC	20 mM pH 9.0 borate buffer containing 30 mM sodium dodecylsulphate; 200 nm	2–200 μg/mL	0.6 μg/mL	1.1–1.76 (n = 6)	[7]
Theophylline, dyphylline /-/drugs	MEKC	10 mM borate buffer at pH 9 and 40 mM SDS as running buffer	0.03–1 μM/mL	0.01 and 0.02 μM/mL	0.60–1.96 (n = 6) 0.72–2.00 (n = 6)	[89]
Caffeine/paracetamol, acetylsalicylic acid/ NeoCitramonum tablets	RAM based SPME CZE	Phosphate buffer at pH 2.5, 6.6, 8.5, 10 and aceton (5% v/v) as an uncharged marker	0.5–100 μg/mL	0.3 μg/mL	1.9	[35]
UA/AA/human urine	CE EC	PMDS microchip; 20 mM acetate buffer pH 5.0; carbon fiber cylindrical electrode vs Ag/AgCl, amperom.	25–500 μM	4.8 μM	0.8–1.6 (n = 3)	[90]
Caffeine/pyroglutamate/ coffee 5.1–5.79 (n=12)	MEKC	50 mM borate buffer at pH 9.5 with 130 mM SDS; 200 nm	4–200 μM	0.23 μM/L	4.2–6.4 (n = 6)	[91]

(*Continued*)

TABLE 3 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation metod	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
UA/AA/human plasma	CE	100 mM/L sodium borate buffer pH 8	10–100mg/L	0.5mg/L	3.9 (n = 10) 5.8(n = 10)	[92]
Caffeine, theobromine, thephylline/-/-	CE	20 mM sodium tetraborate buffer, pH 9.2.; 212 nm	0.40–200 μg/mL 1.20–20 μg/mL 1.7–50 μg/mL	0,12 μg/mL 0.33 μg/mL 0.25 μg/mL	<1,4	[93]
UA/-/chicken plasma and the allantoic fluid of chicken embryos	CE DAD	60 mM MES + 30 mM Tris + 0.001% (w/v) polybrene (pH 6.1); 292 nm	1–200 mg/L	0.2 mg/L	<4.1	[94]
UA/-/plasma	sCZE (short-end injection)	75 mM/L sodium glycylglycine solution pH 9.0; 292 nm	15–150 mg/dL	2 mg/L	<2.5 (C.V.)	[95]
UA/dopamine/serum, urine	CE EC	PDMS microchip; 25 mM MES buffer (pH 5.5); Amperom. at +0.90 V vs. Ag/AgCl (3 MKCl)	15–110 μM	1 μM	–	[96]
Hypoxanthine, UA, xanthine/*p*-aminohippuric acid (PAH) /saliva, urine	CE EC	80 mM/L borax running buffer (pH 9.2); 300μm diameter carbon disc electrode, +950 mV (vs SCE)	$0.01-2 \times 10^{-4}$ M/L	0.50,0.66,0.92 $\times 10^{-6}$ M/L	–	[97]
UA/creatinine, creatine/urine	CE EC	PDMS microchip; 30 mM borate buffer (pH 9.4); gold wire electrode (25 μm diameter) vs Ag/AgCl	0.27–3.07 mM	270 μM	–	[98]
Caffeine/ paracetamol, p-nitro benzyl alcohol, m-nitrophenol and p-nitrophenol/-	MEKC-FTIR	15 mM phosphate buffer at pH 7 and 40 mM SDS	2–10 mM	1.21 mM	–	[99]
UA/cretinine, glucose, AA/urine, serum	CE EC	20 mM phosphate buffer (pH 7.5), amperom. Pt film electrode (10 μm), +1.0 V detection potential (vs. Ag/AgCl)	10–800 μM	2.3 μM	–	[100]

Caffeine, theobromine, theophylline/-/ tea leaf, roasted coffee, coca cola, and theophylline tablets	qCE	15 mM borax buffer; 274 nm	1.6–3.0 μg/mL	3.0 μg/mL (caffeine) 2.1 μg/ml (theo-bromine), 1.6 μg/mL (theo-phylline)	0.33–5.92	[18]
1-MX, 1,7-DMX, caffeine/-/-	CEC DAD	4.0 mM citrate buffer (pH 5.0) containing 80% methanol; 270 nm	2.5–75 μg/mL	0.59 μg/mL; 0.86 μg/mL; 1.11 μg/mL 5.57; 5.94; 5.33 (n = 3)	0.78; 1.66; 0.44 (n = 3)	[101]
Anhydrous caffeine/ thiamine nitrate, acetaminophen, riboflavin, guaifenesin, pseudoephedrine hydrochloride, AA, ethenzamide, DL-methylephedrine HCl, dihydrocodeine phosphate, ibuprofen, noscapine, carbinoxamine maleate, bromhexine HCl/cold medicine	CD-MEEKC	0.81% (w/w) pentane, 6.61% (w/w) 1-butanol, 2% (w/w) 2-propanol, 4.47% (w/w) SDS, and 86.11% (w/w) 10 mM sodium tetraborate solution with 3 mM 2,6-di-*O*-methyl-β-CD	50–200 μg/mL	1.2 μg/mL	0.8–1.9 (n = 3)	[102]
Theobromine, caffeine, theophylline/ chlorogenic acid/yerba mate	MEKC UV	100 mM boric acid (pH 8.5) + SDS to a final concentration of 50 mM; 200 nm	1–150 μg/mL	<1 μg/mL	<2	[16]

(*Continued*)

TABLE 3 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation metod	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
UA/-/biological fluids	CE-C^4D	10 mM 2-morpholinoethanesulfonic acid (MES), 10 mM histidine and 0.1 mM hexadecyltrimethylammonium bromide (CTAB), pH 6.0,	10–500 μM	3.3 μM	<10	[103]
UA/homocysteine, cysteine, reduced glutathione, AA/biological samples	CE EC	100 mM phosphate buffer pH 7.8; platinum disk electrode +1.05V vs. SCE; HDV	1,0–1000 μM	1,6 μM		[104]
UA/AA/-	CE EC	PDMS microchip; acetate buffer of pH 5.5; amperom., carbon fiber microcolumn electrode (diameter 8μm, length 1 mm) vs. Ag/AgCl	5.0 μg/mL–0.40 mg/mL	1 μg/mL		[105]
Caffeine, theophylline/-/rat serum, urine	CE EC	PDMS microchip; 5.0 mM borate solution (pH 9.2) containing 10% (v/v) methanol; 270 nm	6 μM–0.6 mM	4 μM	0.8–5.0 n = 3	[106]
UA/-/serum	CE CL	12 mM Na_2HPO_4 buffer (pH 9.3) containing 0.12 mM AuNP and 0.2 mM luminol.; post-column oxidizer solution was 3 mM NaOH solution containing 35 mM H_2O_2	2.0×10^{-7}– 1.0×10^{-5} M	4.6×10^{-8} M	3.8	[107]
UA/AA/human urine, serum	CE CL	7.5 mL of 0.1 M sodium borate + 250 μl of 0.01 M luminal, pH 9.2, oxidizer solution was prepared by dissolving 250 μl of 0.01 M $K_3[Fe(CN)_6]$ stock solution in 25 mL of 0.2 M NaOH	6.0×10^{-7}– 3.0×10^{-5} M	3.5×10^{-7} M	1.8–3.6 (n = 5)	[108]
UA/AA/human plasma	CE	100 mM/L sodium borate, pH 8	10–100 mg/L	0.5 mg/L	3.1	[109]

UA/-/human serum	CE EC	Cetyltrimethylammonium bromide (CTAB)/2-(N-morpholino)-ethanesulfonic acid (MES) buffer system; carbon fiber microcylinder electrode; 0.80 V vs. Ag/AgCl	5.00×10^{-4} −2.50 $\times 10^{-7}$ M	25 nM	–	[110]
UA/AA, malondialdehyde (MDA)/tears	CE DAD	25 mm borate buffer, pH 10.0, containing 100mm sodium dodecyl sulfate; 290 nm	2–500 μM	0.5 μM	1.7–2.0	[111]
Caffeine,dyphylline, theophylline, theobromine/enprofylline/-	CE	20 mM borate buffer at pH 9.4, 200 nm	1.9–1900 mg/l (caffeine, theophylline), 1.8–1800 mg/l (dyphylline), 2.5–250 mg/l (theobromine)	1.9–2.5 mg/l	0.06–0.22 (n = 5)	[21]
Hypoxanthine/adenosine, inosine/-	CE DAD	30 mM sodium tetraborate, pH 9.4; 259 nm	5–200 μM	3.6 μM	0.33–7.5	[112]
Theobromine, hypoxanthine, theophylline/adenine, guanine allopurinol, deoxyadenosine/-	CE UV	Dynamic pH junction multiplexed CE; 214 nm	0.4–400 μM	8.0×10^{-8} -2.0×10^{-7}M	2.38	[113]
Theophylline, hypoxanthine, xanthine, UA/adenine, guanine/-	CE EC	100 M/l borate buffer (BB, pH 10.0); 300 μm carbon disc electrode at +0.95 V (vs. (SCE)	$0.001–1.0 \times 10^{-3}$ M/L and $0.0025–1.00 \times 10^{-3}$ M/L for hypoxanthine	0.426×10^{-6} M/L (theo-phylline), 0.767 $x10^{-6}$ M/L (hypoxan-thine), 0.417 $x10^{-6}$ M/L (xanthine), 0.363×10^{-6} M/L (UA)	–	[114]

(*Continued*)

TABLE 3 (Continued)

Compound/simultaneously determinate with/sample matrix	Separation metod	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	Intraday RSD% / Interday RSD%	Ref.
Hypoxanthine, xanthine/ 2,8-dihydroxyadenine, allopurinol, oxypurinol, adenine, hippuric acid/urine	CE UV	hypoxanthine, 80 mM SDS dissolved in 60mM sodium tetraborate buffer (pH 8.7); xanthine, 60 mM sodium tetraborate buffer (pH 8.7); 254 nm	hypoxanthine, 18–950 μM, xanthine, 13–1500 μM	5 μM	1.4–6.3	[115]
Dyphylline,theophylline/-/-	MEKC	20 mM tetraborate pH 9.3	0.1–1 mM	0.013 mM (dyphylline), 0.010 nM (theo-phylline)	–	[116]
Caffeine, Theobromine/Catechins/-	MEKC	Phosphate buffer pH 2.5	10–50 μg/mL (caffeine), 50–500 μg/mL (theobromine),	0.80 μg/mL (caffeine), 3.20 μg/mL (theo-bromine)	1.1–7.8 (for all)	[22]
Hypoxanthine, xanthine, UA/allantoin/ serum, plasma	CE UV	20 mM/L sodium tetraborate and 30 mM/L sodium phosphate pH 8.6	0.5–150 μM/L, 10–1500 μM/L for UA	0.3 μM/L (hypoxan-thine), 0.5 μM/L (xanthine), 0.7 μM/L (UA)	–	[117]
Caffeine/sulfacetamide/-	MEKC	15 mM borate buffer, pH 9.3, containing 30 mM SDS and 10% (v/v) ACN. at 215, 234 and 273 nm	5–200 mg/L	1.6 mg/L	–	[118]
Dyphylline,theophylline, caffeine/-/human plasma	CE UV	Tris buffer (20 mM; pH 9.0) with SDS (150 mM); 214 nm	20–200 μM	2, 4, 2 μM	>3.8 >6.5	[192]

MEKC, micellar electrokinetic capillary chromatography; *RAM,* restricted-access media; *CD-MEEKC,* cyclodextrin-modified microemulsion electrokinetic; chromatography, *CL,* chemiluminescence; *EC,* electrochemical detection; *SDS,* sodium dodecyl sulfate.

TABLE 4 Electrochemical Biosensors Based on Urate Oxidase (UOx)

Compound/ simultaneously determinate with/ sample matrix	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	RSD%	Ref.
UA/-/urine	amperometric biosensor based on UOx–peroxidase coupled enzyme system; phosphate buffer (pH 7.5; 50 mM);	0.1–0.5 μM	0.1 μM	2.96	[119]
UA/-/-	PS/UOx/GE; oxygen-saturated phosphate buffer solution (PSB, 10mM, pH 8.5); CV	5–105 μM	–	2.8 (n = 10)	[120]
UA/-/blood samples	UOx/ZnS QDS/L-cys/Au; pH 6.9 phosphate buffer; CV	5.0×10^{-6} −2.0×10^{-3} M/L	2.0×10^{-6} M/L	1.3–4.4 (n = 3)	[121]
UA/-/ dialysate samples in rat striatum	UOx/MWCNTs–SnO_2/GCE; amperom.	1.0×10^{-7} −5.0×10^{-4} M/L	5.0×10^{-8} M/L		[122]
UA/-/urine	UOx-CS/Hb-CS/GCE; 0.05 M phosphate buffer (PBS) pH 7; CV	2.00–30.0 μM	0.85 μM	1.36–4.23 (n = 5)	[123]
UA/-/-	UOx Ir–C electrode; pH 7 phosphate buffer solution (PBS); amperom.	0.1–0.8 mM	0.01 mM	–	[124]

Uox, urate oxidase; *UA,* uric acid; *QDS,* quantum dots;, *MWNTs,* multiwalled carbon-nanotubes; *Hb,* hemoglobin; *CS,* chitosan.

biosensor based on functionalized multi-wall carbon nanotubes (MWCNTs) with tin oxide (SnO_2) nanoparticles. The LR was 1.0×10^{-7}–5.0×10^{-4} mol/L (see Table 4).

Biosensors with Xanthine Oxidase (XOD)

Xanthine oxidase (125–141) (Table 5) catalyzes the oxidation of hypoxanthine to xanthine (Equation (5)) and can further catalyze the oxidation of xanthine to UA (Equation (6)):

$$\text{Hypoxanthine} + O_2 \xrightarrow{\text{XOD}} \text{Xanthine} + H_2O_2 \tag{5}$$

$$\text{Xanthine} + O_2 \xrightarrow{\text{XOD}} \text{Uric Acid} + H_2O_2 \tag{6}$$

TABLE 5 Electrochemical Biosensors Based on Xanthine Oxidase (XOD)

Compound/simultaneously determinate with/sample matrix	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	RSD%	Ref.
Xanthine/-/-	XOD-CD-CMC-ADA-Au; 0.1M sodium phosphate buffer, pH 7.0	300 μM–10.4 mM	200 μM	–	[125]
Xanthine/-/-	XOD/Nano-$CaCO_3$ (E = 0,55 V) and XOD/HRP/Nano-CaCO3 (E = –0,05 V); phosphate buffer (0.05 M) containing 2 mM EDTA, pH 7.5	2×10^{-6}–$2,5 \times 10^{-4}$ M (anodic detect), 4×10^{-7}–5×10^{-5} M (catodic detect)	2×10^{-6} M (anodic detect), 1×10^{-7} M (catodic detect)	–	[126]
Xanthine	XOD/LDHs/GCE Amperom. (E = 0,55 V); phosphate buffer solution (0.05 M, pH 7.5)	1×10^{-6}–2×10^{-4} M	1×10^{-7} M	4.5	[127]
Hypoxanthine/-/-	GA–BSA–XOD–nAu–CPE; 0.05 M/l phosphate buffer of pH 7.4	5.0×10^{-6}–2.5×10^{-5} M/l	2.2×10^{-7} M/l	3.9 (n = 10)	[128]
Hypoxanthine/-/ cell culture media	Nafion/XOD/PPh/CFME; phosphate buffer solution (0.1 M, pH 7.0); +0.60 V (vs Ag/AgCl)	5.0 μM-1.8 mM	1.5 μM	–	[129]
Hypoxanthine/ glucose, lactate, L-glutamate/ brain	Nafion/XOD-NRDS/CE in FIA; 0.1 M phosphate buffer pH 6.9; CV	5.0×10^{-7}–$5,0 \times 10^{-5}$ M/L and 1.0×10^{-4}–1.2×10^{-3} M/L	2×10^{-7} M/L	4.6	[130]
Hypoxanthine/-/-	XOD/MV-SWy-2-CPE; phosphate buffer solution at pH 7.0.; CV	1 μM-0,4 mM	0,8 μM	–	[131]
Xanthine/-/-	XOD/laponite/GCE; phosphate buffet (PBS) at pH 7.0; CV	3.9×10^{-8}–2.1×10^{-5} M	1.0×10^{-8} M	–	[132]
Xanthine/-/-	XOD/poly-TTCA/Au; 0.1 M phosphate buffet at pH 7.5; CV	anodic 5.0×10^{-6}–1.0×10^{-4} M, catodic 5.0×10^{-7}–1.0×10^{-4} M	anodic 1.0×10^{-6} M, catodic 9.0×10^{-8} M	anodic 7.5, catodic 6.7	[133]

Xanthine, hypoxanthine /-/-	XOD/GCPE; Phosphate buffer (0.05 M, pH 7.0), chronoamperom.	5.0×10^{-7}–4.0×10^{-5} M 2.0×10^{-5}–8.0×10^{-5} M	1.0×10^{-7} M; 5.3×10^{-6} M	–	[134]
Xanthine/-/-	XOD/CNT/GCE; phosphate buffer solution (pH 7.0); CV	0.1 μM–6 μM	0.08 μM	–	[135]
Xanthine/-/-	SG/XOD/CNT/GCE; phosphate buffer solution (pH 7.0); CV	0.2–10 μM	0.1 μM	–	[135]
Hypoxanthine/-/-	Os-gel-HRP/XOD/GCE; phosphate buffet (PBS) at pH 7.0; CV	0.5–80 μM	0.2 μM	–	[136]
Theophylline/-/-	microbial XOD electrode; 0.1 M phosphate buffet at pH 7.5;	2×10^{-7}–5×10^{-5} M	2×10^{-7} M	–	[137]
Xanthine/-/-	XOD PPY/Pt; buffer: 0.025 M phosphate and 0.1M lithium perchlorate pH 7.4; amperom.	1.0–400 μM	1 μM	–	[138]
Xanthine/-/-	XOD-SOD-HRP (xanthine oxidase, superoxide dismutase and peroxidase) co-immobilized in a single sol-gel matrix; sodium phosphate buffers (10 mM, pH 7.4); fluorescence	0–3.5 μM	0.02 μM	–	[139]
Hypoxanthine/-/ fish	XOD- UM/Hx–S/O_2; 0.05 M phosphate buffer pH 7,6; CV	1 μM–1 mM	0.4 μM	–	[140]
Xanthine/-/-	XOD-ADA-AuPolyCD; 20 mM sodium phosphate buffer, pH 7.0; CV	310 μM–6.8 mM	150 μM	–	[141]

XOD, xanthine oxidase; *CD,* β-cyclodextrin; *CMC,* carboxymethylcellulose; *ADA,* 1-adamantanyl, *HRP,* horseradish peroxidase; *LDHs,* layered double hydroxides; *BSA,* bovine serum albumin; *nAu,* nanocrystal gold; *CFMEs,* carbon fiber microelectrodes *PPb,* electropolymerized phenol; *NRDs,* neutral red-doped silica; *MV,* methyl viologen (paraquat, *N*,*N*-dimethyl-4,4-bipyridinium); *SWy-2,* sodium montmorillonite; *poly-TTCA,* (poly-5, 2': 5', 2"-terthiophine-3-carboxylic acid), *UM/Hx-S/O_2*, unmediated Hx sensor in hydrogen peroxide.

The biosensors were working in phosphate buffer in pH ranging from 6.8 to 7.6. The LR covered millimole (mM) to micromole (μM) concentrations.

The hypoxanthines (128, 129, 131, 136, 140) and xanthines (125–127, 132, 133, 135, 138, 139, 141) were analyzed alone. However Zhang et al. (130) determined hypoxanthines beside glucose, lactate and L-glutamate in rat stratium.

For more accurate detection of xanthines, Salinas-Castillo et al. (139) have developed a trienzymatic system, based on coupled XOD–SOD–HRP (Peroxidase – HRP, superoxide dismutase –SOD, xanthine oxidase - XOD) system in a single sol–gel matrix to prepare a fluorescent biosensor. The LOD was 0.02 μM which largely improves the sensitivity of the current xanthine biosensors however the LR (0–3.5 μM) requires diluting the sample. Peroxidases were also used in the double system by Shan et al. (126) and Mao et al. (136) with wider LR.

The interesting biosensor for theophylline build on microbial XOD was proposed by Stredansky et al. (137). It showed a low LOD of 2×10^{-7} M and LR up to 5×10^{-5} M.

Biosensors with Theophylline Oxidase ThO

The catalytic mechanism of ThO involves oxidation of theophylline to 1,3-dimethyluric acid; the reduced enzyme can then be re-oxidized by cytochrome C or by other electron acceptors that completing the biocatalytic cycle (142,143) as clearly seen on equation 7:

$$\text{Theophylline} + 2\text{ferricytochrome } c \xrightarrow{\text{Tho}} 1,3\text{-dimethyluric acid} + 2\text{ferrocytochrome } c \quad (7)$$

The biosensors were working in phosphate buffer in pH 7.0. The LR and LOD covered millimolar concentrations. The lowest LOD (0.02 mM) was obtained by Ferapontova at al. (142) with a sensitivity of 52 mA/M cm^2 (Table 6).

NON-ENZYMATIC BIOSENSORS

Non-enzymatic devices were the largest group among the biosensors (144–182) (Table 7). Different techniques were used to prepare them such as physical adsorption, cross-linking, covalent bonding, and gel or membrane entrapment. Among these techniques, electrogenerated polymerization has been receiving a great attention due to a number of advantages. The main advantage is that the immobilizing process can be controlled by adjusting

TABLE 6 Electrochemical Biosensors Based on Theophylline Oxidase (ThOx)

Compound	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	RSD%	Ref.
Theophylline	ThOx in Os-redox-polymer on GE, 0,15 V; 10 mM phosphate buffer containing 0.15 M NaCl (PBS), pH 7.0	0.02–0.6 mM	0.02 mM	–	[142]
Thephylline	ThOx/DDAB on GE, 0,15V; 10 mM phosphate buffer solution, containing 0.15 M NaCl (PBS), pH 7.0	0.2–8 mM	0.2 mM	–	[142]
Theophylline	ThOx/SAM-modified Au, 0,15 V; 10 mM phosphate buffer solution, containing 0.15 M NaCl (PBS), pH 7.0	2–3 mM	2 mM	–	[142]
Theophylline	ThOx on GE, 50 mM phosphate buffer at pH 7; −0.1 V and +0.3 V vs Ag|AgCl (0.1M KCl)	0.2–2 mM	0.2 mM	–	[143]

TbOx, theophylline oxidase; *Os,* osmium; *DDAB,* didodecyldimethylammonium bromide; *SAMs,* self-assembled monolayers.

the electrochemical parameters, so the thickness, permeation and charge transport characteristics of the polymeric film is known whatever the size or geometry of the electrode surface are.

The most common analyte quantified using non-enzymatic biosensors was UA (144–166, 169, 172, 174, 176, 181, 182). UA was very often analyzed simultaneously with AA. It is a problem to separate the signals (145, 147–148, 150–153, 155–157, 159–164, 166, 176, 181). The very good separation of UA from AA was demonstrated by Liu et al. (155). Using a glassy carbon electrode (GCE) modified with 4-(2-pyridylazo)-resorcinol (PAR) polymer film, the authors obtained the LR values of 1.0×10^{-8}–5.0×10^{-5} mol/L and LOD 1.0×10^{-9} mol/L.

Other simultaneous determinations of UA were with dopamine (DA) (145, 154, 156, 160, 162, 163, 166, 181, 182) or with ascorbic acid (AA) and dopamine (145, 156, 160, 162, 163, 166, 181), norepinephrine (146), epinephrine (159, 164), tryptophan (150), paracetamol (151), xanthine (144, 172) and hypoxanthine (175). Xie et al. (174) determined UA and xanthine additionally with hypoxanthine.

The caffeine (167,168,177) and theophylline (170,173,178) values of LR and LOD were measured with the low millimolar to micromolar range of concentrations. The exception was the work of Liu et al. (178) where authors obtained on platinum nanoparticles (Pt_{nano}) coated with a multiwalled carbon nanotube (MWCNTs)-1-octyl-3-methylimidazolium hexafluorophosphate ((OMIM)(PF_6)) composite material film (MWCNTs-Pt_{nano}-(OMIM)(PF_6)) and deposited on a glassy carbon electrode (GCE) the LR of 1.0×10^{-8}–1.0×10^{-5} M and the LOD estimated to be 8×10^{-9} M.

TABLE 7 Non-Enzymatic Biosensors Used in Analysis of Xanthines

Compound/simultaneously determinate with/sample matrix	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	RSD%	Ref.
Caffeine/paracetamol/ aqueous media	BDD electrode; acetate buffer at pH 4.5; peak oxidation potentials of 0.80 V and 1.37 V vs. Ag/AgCl (3.0 mol/L KCl);	3.0×10^{-7}–9.1×10^{-5} M/L	3.5×10^{-8} M/L	–	[6]
Xanthine,UA/-/urine	polyacrylonitrile (PAN)-based carbon fiber; sodium phosphate buffer, pH 7.4; FSV	0.5–6 μM, 0.5–4.5 μM (UA)	1 μM, 500 nM (UA)	–	[144]
UA/dopamine,AA/-	DNA/Pp-ABSA/GCE; 0.1M phosphate buffer (PBS) containing 0.5 M KCl, pH 7.0; DPV	0.38–23 μM	0.19 μM	–	[145]
UA/norepinephrine,AA/-	poly-CCA GCE; CV; phosphate buffer (pH 6.0)	1.0–200.0 μM	0.5 μM	–	[146]
UA/AA/-	β-CD/SPNAANI/GCE; 0.1 M/L phosphate buffer (pH 7.4, 0.1 M/L KCl); DPV	1.0×10^{-5}–3.5×10^{-4} M/L	2.7×10^{-6} M/L	1.45 (n = 10)	[147]
UA/AA/urine	PAN-ABSA/GCE; 0.1 M/l phosphate buffer PBS (pH 6.8); DPV, CV	50–250 μM/L	12 μM/L	3,1–3,6 n = 6	[148]
UA/-/urine	poly(3-acetylthiophene)/GCE; 0.1 M phosphate buffer (PBS) at pH 7.2; SWV	1.25×10^{-5}–1.75×10^{-4} M	5.27×10^{-7} M	2,95 (n = 5)	[149]
UA/tryptophan,AA/-	Fe(3+)Y/ZCME; phosphate buffer pH 3.5; DPV	0.3–700 μM	0.08 μM	3,68	[150]
UA/AA, paracetamol/-	DTB–PET/MPx-11; sodium phosphate buffer solution (PBS); CV	5–150 μM	2 μM	–	[151]
UA/AA/urine	Fc^{+}–thioglycollate modified gold electrode; phosphate buffer solution (PBS, pH 7.0); CV/DPV	1.0×10^{-6}–5.0×10^{-4} M	1.0×10^{-7} M	1,8 (n = 10)	[152]
UA/AA/urine	Qu/WGE; phosphate buffer solutions (PBS, 0.1 M); pH 7.0; amperom.	1–50 μM	1.0 μM	–	[153]
UA/dopamine/-	AuNPs-P3MT/GCE; phosphate buffer solutions (PBS, 1/15 M/L) pH 7.0; CV, DPV	1.0×10^{-6}–3.2×10^{-5} M/L	1.7×10^{-7} M/L	2.63 (n = 10)	[154]

UA/AA /urine	p-PAR/GCE; phosphate buffer (PBS, 0.05 M/L); pH 2.0; CV, DPV	1.0×10^{-8}–5.0×10^{-5} M/l	1.0×10^{-9} M/l	1.9–2.7	[155]
UA/dopamine, AA/-	PAA-MWNTs/GCE; 0.1 M phosphate buffer (pH 7.4);CV, DPV	0.3–10 μM	110 nM	–	[156]
UA/AA /urine	o-AP/GCE; phosphate buffer solution (0.1 M, pH 7.0); d.c. amperometry	2–20 μM	0.3 μM	2.5 (n = 10)	[157]
UA/-/-	OMC–Fc/GC; 0.1 M phosphate buffer solution (pH 7.3); CV	60–390μM	1.8 μM	2.0–2.1	[158]
UA/epinephrine, AA/-	poly(caffeic acid)/GCE; 0.15 M/l phosphate buffer pH 7.7; CV	5.0×10^{-6}–3.0×10^{-4} M/L	6.0×10^{-7} M/L	2.59–4.55 (n = 5)	[159]
UA/dopamine/AA /-	PtAu hybrid film/GC; potassium hydrogen phthalate (KHP) buffer of pH 4.0; CV, DPV	0.021–0.336 mM	0.021 mM	<2,0 (n = 10)	[160]
UA/AA /urine	PS-*b*-PAA NEE; 0.10 M phosphate buffer (PBS) pH 6.86; DPV, CV	0.25–50 μM	0.04 μM	–	[161]
UA/dopamine, AA/-	EBT GC; phosphate buffer solution (pH 4.0); DPV	10–130 μM	1,0 μM	–	[162]
UA/dopamine, AA/-	PPy/SWNTs/GCE; Phosphate-buffered saline (PBS; 0.1 M); CV, DPV	2–100 μM	0,74 μM	–	[163]
UA/epinephrine, AA/urine	Nano-Au/PPyox/GCE; pH 7.0 phosphate buffer solution (PBS); DPV, CV	0,05–28 μM	0,012 μM	2,6–3,2 (n = 15)	[164]
UA/-/-	Poly-BAHHFP; Britton-Robinson buffer pH 1.8; DPV	0.5–100 μM	0.1 μM	–	[165]
UA/dopamine, AA/-	OBMGCE; phosphate buffer solution (pH 8.0); DPV	12.5–400.0 μM	0.4 μM	–	[166]
Caffeine/-/-	piezoelectric caffeine sensor –MIP-PPy/gold electrode/quartz crystal/caffeine; phosphate buffer (0.1 M, pH 7)	0.1–10 mg/mL	4.66×10^{-6} mg/mL (0.024 μM)	–	[167]
Caffeine/-/-	MIP–PMAA/PVC piezoelectric quartz sensor; buffer solution (pH 8)	1×10^{-9}–1×10^{-3} mg/mL	3.76×10^{-11} mg/mL	–	[168]

(*Continued*)

TABLE 7 (Continued)

Compound/simultaneously determinate with/sample matrix	Detection method/details	Linear range	Sensitivity (LOD or LOQ)	RSD%	Ref.
Hypoxanthine, xanthine, UA/-/blond plasma, urine	NSPE; phosphate buffer solution (PBS) pH 7.0; SWV	4–30 μM, 2–40 μM, 2–40 μM	0.34 μM, 0.07 μM,0.42 μM	0.6, 1.3, 3.8	[169]
Theophylline/-/-	MWCNT GCE SCE; 0.1 M/L phosphate buffer at pH 5.8; CV	3×10^{-7}–1×10^{-5} M/L	5×10^{-8} M/L	3.3 (n = 7)	[170]
Xanthine/-/human serum	CNTs/GCE; HAc-NaAc buffer pH 5.2; anodic stripping voltammetry	$2{,}0 \times 10^{-7}$–$2{,}0 \times 10^{-5}$ M	$4{,}0 \times 10^{-9}$ M/L	–	[171]
UA, xanthine, hypoxanthine/ allopurinol/-	g-PGEe; 0.1 M acetate buffer (pH 4.8); DPV	25 nM–2 μM	25 nM	–	[172]
Theophylline/-/serum	RNA aptamer-based biosensor; HEPES buffer, pH 7; CV, DPV	0,2–10 μM	0,2 μM	–	[173]
UA, xanthine, hypoxanthine/-/-	SWNTE/IUTCPE; (pH 5.72) Britton-Robinson (B.R.) buffer; CV, DPV	$1{,}0 \times 10^{-7}$–$8{,}0 \times 10^{-6}$(UA), $2{,}0 \times 10^{-7}$–$1{,}0 \times 10^{-4}$ (xanthine), $8{,}0 \times 10^{-7}$–$2{,}0 \times 10^{-5}$ M (hypoxanthine)	$8{,}0 \times 10^{-8}$ (UA), $1{,}46 \times 10^{-7}$ (xanthine), $5{,}62 \times 10^{-7}$ M (hypoxanthine)	–	[174]
Hypoxanthine/-/-	TiO_2-CPE; pH 7.0 phosphate buffer (0.1 M/L); CV	2.0×10^{-7}–5.0×10^{-5} M/L	5.0×10^{-8} M/L	<5	[175]
UA/AA/-	Fullerene-C_{60} GCE; phosphate buffer (0.1 M/L) pH 7.2; LSV	0,5–700 μM	0,2 μM	–	[176]
UA/AA/-	Fullerene-C_{60} GCE; phosphate buffer (0.1 M/L) pH 7.2; OSWV	0,5–800 μM	0,12 μM	–	[176]
Caffeine/-/-	BQMCPE; pH 6; CV, SWV	0–0.5 mM/L and 0.5–8 mM/L	0.3 μM/L and 5.1 μM/L	–	[177]

Theophylline/-/-/medicine tablets/green tea	MWCNTs-Pt_{nano}-[omim][PF_6]/GCE SCE; phosphate buffer (PBS 0.1 M/L) pH 3	1.0×10^{-8}–$1{,}0 \times 10^{-5}$ M	8.0×10^{-9} M	2,13 (n = 4) medicine tablets, 3,1 (n = 5) green tea	[178]
Theophylline, theobromine, caffeine, xanthine/-/-	BBD/GCE SCE; Britton-Robinson buffer pH 1.8; CV	1–400 μM (theophylline), 1–100 μM (others)	–	0,3–3,5	[179]
Xanthine/-/-	PAR GCE copper; Britton-Robinson buffer pH 7.25; DP-ASV, CV	19.9–166 nM (peak I), 0.24–17.2 nM (peak II)	0.138 μg/L	2.8 (n = 5)	[180]
UA/dopamine, AA/-	PGE-DA; phosphate buffer, pH 10; DPV	2.5×10^{-6}–2.0×10^{-5} M/L	1.4×0^{-6} M/L	–	[181]
UA/dopamine/-	GNP/Ch/GCE; phosphate buffer (PBS) pH 7.0; DPV	1.2–100 μM	0.6 μM	–	[182]

UA, uric acid; *AA,* ascorbic acid; *Pp-ABSA,* poly(p-aminobenzensulfonic acid); *CCA,* polycalconcarboxylic acid; *β-CD, β*-cyclodextrin; *SPNAANI,* sulfanilic acid (*p*-ASA) and N-acetylaniline; *Fe(3+)Y/ZCME,* iron(III) doped zeolite modified carbon paste electrode; *DTB–PET/MPx-11,* microperoxidase-11 (MPx-11) self-assembled monolayer (SAM) 2-(2-mercaptoethylpyrazine) (PET) 4,4′-dithiodibutyric acid (DTB), *Qu/WGE* quercetin-modified wax-impregnated graphite electrode; *pPAR,* 4-(2-pyridylazo)-resorcinol; *PAA,* poly(acrylic acid); *MWNTs,* multiwalled carbon-nanotubes; *o-AP, o*-aminophenol; *OMC,* ordered mesoporous carbon functionalized with ferrocenecarboxylic acid (Fc); *PS-b-PAA,* polystyrene-block-poly (acrylic acid); *NEE,* nanoelectrode ensembles; *EBT,* eriochrome black T; *Ppy,* polypyrrole; *SWNTs,* single-walled carbon nanotubes; *Ppyox,* overoxidized polypyrrole; *BAHHFP,* 2,2-bis(3-amino-4-hydroxyphenyl)hexafluoropropane; *OBMGCE,* oracet blue modified glassy carbon electrode; *MIP,* molecularly imprinted; *PMAA,* co-polymerizing methacrylic acid; *NSPEs,* nontronite-coated screen-printed carbon electrodes; *PGE,* pyrolytic graphite electrodes; *SWNT/IUTCPE,* single-wall carbon nanotube film electrode constructed on the surface of inlaying ultra-thin carbon paste electrode; *BQMCPE,* 1,4-benzoquinone modified carbon paste electrode; *BDD,* boron-doped diamond; *GNP/Ch,* gold nanoparticle/choline.

Ferapontova et al. (173) have been successful using a RNA aptamer-based electrochemical biosensor in theophylline analysis.

The biosensors were working in phosphate buffer in pH from 6.0 to 8.0, however more acidic conditions (under pH 5) were not rare (150, 160, 162, 165, 171, 178). It is important to point out the work of Da Silva et al. (181) where the authors successfully separated UA form DA on pyrolytic graphite electrodes (PGE) modified into dopamine solutions using phosphate buffer solutions pH 10 as supporting electrolyte.

UV SPECTROPHOTOMETRY

In only three articles over this period of time UV spectrometry was used. Kelani et al. (193) applied ratio–spectra zero-crossing first-derivative spectrometric and two chemometric procedures: PLS (partial least-squares) and PCR (principal component regression) as supporting techniques helpful in accurate determination of 8-chlorotheophylline and caffeine beside chlorphenoxamine hydrochloride. The LR and LOD parameters were as follows: 4–20 μg/mL (LR), 0.24 μg/mL (caffeine), 0.34 μg/mL (8-chlorotheophylline) with ratio-spectra zero-crossing first-derivative spectrometric method and 4–25 μg/mL (LR), 0.13 μg/mL (caffeine), 0.15 μg/mL (8-chlorotheophylline) with PLS and PCR methods.

Llorent-Martínez et al. (55) used a single continuous-flow solid-phase UV spectrophotometric sensing system for determination of caffeine, theophylline and theobromine in food products, beverages and pharmaceutical preparations. The results were comparable to those obtained using a HPLC method.

Sentürk et al. (65) determined the theophylline and ephedrine HCl content in tablets using a simple UV detector to measure the solution absorbance.

CONCLUSIONS

The aim of this review was to present an overview of the advances of analytical techniques used for determination of methylxanthines and their analogues for the last 10 years. The most commonly employed method is HPLC. The objects of separation were rarely individual components, most frequently there were simultaneous determination of multiple constituents in body fluids (serum, plasma, urine), complex medicines, plant products (e.g. tea leaves, coffee, soft drinks) or sewage sludge. The analysis was usually carried out in reversed phase mode with classical 4.6 cm i.d. C_{18} columns. Very often, pre-columns (guard columns) were used to protect the working column. Typical analysis was carried out using a gradient elution. The mobile phase usually consists of water miscible organic solvents such as

acetonitrile or methanol and water or buffer solutions (pH neutral or acidic). The most commonly used modes of RPLC detection are UV spectrometry at a single wavelength or multiple wavelengths with a diode array detector (DAD), mass spectrometry with electrospray ionization (ESI-MS/MS) or rarely electrochemical detection.

It is interesting that the introduced and very often used in the last years UHPLC technique still has not found beneficial application in the analysis of methylxanthines (except few reports concerning determination of caffeine), but in our opinion it will be more used in the near future.

The next commonly employed method for xanthine determination is CE. CZE, which is the simplest CE mode, has been mostly applied to the separation of UA and UA with ascorbic acid (AA); and in a lesser extent to the determination of caffeine, hypoxanthine, xanthine and theophylline. The analytes was successfully separated by borate or phosphate buffer under basic conditions, although other buffers, such as acetate and Tris were also used. Usually UV detection is the most frequently used detector. However the concentration sensitivity of CE with UV detection cannot usually go below the micromolar level due to the short optical path length and small injection volumes. This is often insufficient to detect trace levels of metabolites in real samples; therefore a more sensitive detection mode is required such as electrochemistry or MS.

MEKC was the best electrophoretic solution for simultaneous determination of multiple xanthine constituents in body fluids, in drugs or in plant products. The most widely used buffers were borate or phosphate salts with SDS (sodium dodecyl sulfate) anionic micelles.

The rapid separation and sensitive determination of methylxanthines were obtained in PDMS microchannel electrophoresis integrated with electrochemical detection. Due to the known limitations of PDMS (extreme hydrophobicity and unstable and/or poorly controlled EOF), many works reported the use of PDMS with surface modified by appropriate coatings. Reversed EOF and thus rapid and efficient separation of methylxanthines was obtained after coating PDMS with poly(allylamine) hydrochloride, poly(diallyldimethylammonium chloride), chitosan or chitosan-DNA.

Other chromatographic methods, i.e., TLC or GC were rarely used for xanthine detrmination.

It is very important to point out the critical importance of sample preparation in xanthine determinations. SPE methods are very good solutions for this problem. The SPE method has several advantages: speed, reduced labor, better recoveries, improved sample-to-sample consistency, significantly reduced solvent consumption and waste production, ruggedness and reliability. However, due to the relatively high polarity of methylxanthines, these compounds are often located in aqueous solutions that require the use of typical apolar C_{18} cartridges that often do not give acceptable results. That is why there were only few reports concerning simple SPE methods. Special

extraction methods such as drop-to-drop solvent microextraction, subcritical water extraction, molecularly imprinted polymer and restricted-access media SPME were used. Another simple solution would be to perform SPE with Oasis® cartridges (HLB or MAX) that provide a high capacity for polar compounds.

Amperometric sensors and biosensors for the specific determination of methylxanthines have attracted a great attention due to their analytical performances. Electrochemical sensors provide, among other advantages, low LOD, a wide LR, a good stability and reproducibility. The measurements are made directly on the raw sample, in real time. The sensor produced electrical signal is directly converted into a measured analytical concentration. The measurement does not require any special long-lasting preparatory procedure or prior treatment with expensive materials.

Enzymatic biosensors are becoming more popular; enzymes are excellent analytical reagents due to their specificity, selectivity, efficiency and allow obtaining correct results with great accuracy and sensitivity. However the small number of publications with enzymatic biosensors is generally caused by their high cost.

The natural methylxanthines: caffeine, xanthine, hypoxanthine, UA and especially their metabolites, have a large share in the current researches. It shows that they are in the strict area of interest of many research groups and has important practical and the scientific value.

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