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Methylxanthines: properties and determination in various objects

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Abstract. Published data on the properties and determination of caffeine, theophylline, theobromine and some other methylxanthines in various objects are surveyed and described systematically. Different sample preparation procedures such as liquid extraction from solid matrices and liquid – liquid, supercritical fluid and solid-phase extraction are compared. The key methods of analysis including chromatography, electrophoresis, spectrometry and electrochemical methods are discussed. Examples of methylxanthine determination in plants, food products, energy beverages, pharmaceuticals, biological fluids and natural and waste waters are given. The bibliography includes 393 references.

I. Introduction

Methylxanthines, which are represented, in particular, by naturally abundant caffeine, theophylline and theobromine, play an important role in biological processes. These compounds are present in tea and other plant leaves, coffee beans, cocoa beans and cola seeds (nuts).[1–8] They exert an excitatory effect on the central nervous system, promote the psychical and physic activities of organisms, stimulate heart and kidney functioning and inhibit the bronchial muscle tone; therefore, they are widely used in modern pharmaceutical practice.[9–16]

Safe and efficient use of the pharmaceutical agents containing methylxanthines requires thorough dosage control, as even minor disruption may cause adverse effects. Excessive intake of methylxanthines has negative consequences (poisoning, nervous trembling, tachycardia, *etc.*), and in some cases, it can cause infertility, malignant growth or even death.[15,17,18] Since the ranges of therapeutic and toxic concentrations of these compounds do not differ much, it is necessary to thoroughly control the body's level of methylxanthines. For example, the therapeutic effect of theophylline is manifested when its blood serum level is $10-20\ \mu g\ ml^{-1}$, and the toxic effect may already start at $>20\ \mu g\ ml^{-1}$ (Ref. 17). According to the rules of the International Olympic Committee, a sportsman is considered to take stimulants if the urine concentration of caffeine is $>12\ \mu g\,ml^{-1}$ (Refs 18 and 19) or the concentration of theophylline, the major caffeine metabolite, is $5\ \mu g\,ml^{-1}$ (Refs 19 and 20). In view of these facts and due to more and more frequent cases of counterfeit medications on sale, there is increasing demand of fast and reliable means for identification and quantification of methylxanthines in biological fluids and pharmaceuticals.

Despite the extensive use of methylxanthines in medicaments, the mechanisms of their action *in vitro* and *in vivo* are still not entirely clear, and this accounts for a large number of publications devoted to determination of these compounds and their metabolites in various biological fluids.[21–29] According to a review,[30] considerable attention is paid to studying the pharmacokinetics and pharmacodynamics involving methylxanthines.

It is also necessary to determine methylxanthines in food items, for example, in chocolate, various energy beverages, whose stimulating effect is mainly due to caffeine they contain. The average daily intake of caffeine for an adult is 1 to 829 mg.[31] The governments of many countries are concerned about this high caffeine intake and take measures toward the control of its content in food products. For example, the US Food and Drug Administration established [32] the maximum permissible concentration of caffeine in energy beverages at 200 mg $litre^{-1}$.

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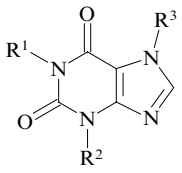
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It is highly important to determine the caffeine content in waters, as it can serve as an indicator of water quality.[33–40]

Thus, determination of methylxanthines in various specimens (ranging from medicaments to biological fluids and waters) is a topical task, and this is evidenced by numerous recent publications on this subject. A number of reviews deal with the biosynthesis of caffeine and other methylxanthines in plants, their pharmacological properties and the impact on the human organism.[1,9–13,21] The present review summarizes the published data of the last 15 years on properties of methylxanthines and methods of their isolation, preconcentration and determination in plants, food products, energy beverages, medicaments, biological fluids and waters.

II. General characteristics and properties of methylxanthines

Methylxanthines are purine alkaloids. Purine is a fused heterocyclic system comprising annulated pyrimidine and imidazole rings. In nature, purine alkaloids are represented by methyl derivatives of xanthine (**1**). The most important of these (compounds **2**–**7**) are characterized in Tables 1 and 2.



1–**7**

Methylxanthines are sparingly soluble in water and ethanol, they have low Hansch parameters [octanol–water partition coefficients (*P*), Table 2]. Theophylline and theobromine are amphoteric compounds whose acidic properties are due to the hydrogen atoms of the imide groups in positions 1 or 7, while the basic properties are due to the nitrogen atom in position 9. The difference between the acidic properties of these compounds is utilized for their identification. Theobromine is soluble in alkalis (NaOH), while theophylline is soluble not only in alkalis but also in aqueous ammonia. Unlike theohylline and theobromine, caffeine is not acidic and does not form insoluble salts with silver, cobalt, copper or iron ions.[15]

1. Historical background

The chemistry of purine alkaloids is usually thought to start in 1819 when Runge isolated a new compound from coffee beans and called it caffeine.[42] In 1827, Oudry isolated a new

Table 1. Compositions and molecular masses of xanthine (**1**) and methylxanthines (**2**–**7**).

Compound	Traditional name	R^1	R^2	R^3	Molecular formula	Molar mass /g mol^{-1}	IUPAC name
1	Xanthine	H	H	H	$C_5H_4N_4O_2$	152.11	3,7-dihydropurine-2,6-dione
2	Theophylline (1,3-dimethylxanthine)	Me	Me	H	$C_7H_8N_4O_2$	180.16	1,3-dimethyl-3,7-dihydropurine-2,6-dione
3	Paraxanthine (1,7-dimethylxanthine)	Me	H	Me	$C_7H_8N_4O_2$	180.16	1,7-dimethyl-3,7-dihydropurine-2,6-dione
4	Theobromine (3,7-dimethylxanthine)	H	Me	Me	$C_7H_8N_4O_2$	180.16	3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione
5	Caffeine (1,3,7-trimethylxanthine)	Me	Me	Me	$C_8H_{10}N_4O_2$	194.19	1,3,7-trimethyl-3,7-dihydropurine-2,6-dione
6	Diprophylline [7-(2,3-dihydroxypropyl)theophylline]	Me	Me	$CH_2CH(OH)CH_2OH$	$C_{10}H_{14}N_4O_4$	254.24	7-(2,3-dihydroxypropyl)-1,3-dimethyl-3,7-dihydropurine-2,6-dione
7	Pentoxyphylline [1-(5-oxohexyl)-theobromine]	$(CH_2)_4Ac$	Me	Me	$C_{13}H_{18}N_4O_3$	278.31	3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydropurine-2,6-dione

Table 2. Some properties of methylxanthines.

Methylxanthine	m.p. /°C	Solubility	log*P* (see [a])	pK_a (see [b])	pK_b (see [b])
2	270–274	poorly soluble in cold water (1 : 180), better soluble in hot water (1 : 85), soluble in acids and alkalis	−0.2	8.8	14.2
3	294–296	insoluble in alcohols or ethers; soluble in hot water	−0.6	–	–
4	290–295	very poorly soluble in water (1 : 700), soluble in acids and alkalis	−0.7	10.0	13.9
5	235–238	poorly soluble in cold water (1 : 60), readily soluble in hot water (1 : 2), sparingly soluble in ethanol (1 : 50), soluble in $CHCl_3$	−0.1	14.0	14.2
6	158–163	moderately soluble in water (1 : 10) and ethanol	−1.10	–	–
7	103–106	soluble in water	0.3	–	–

[a] Hydrophobicity parameters were calculated using the log*P* programme (@ACD, Canada).
[b] The negative logarithms of the acidity (pK_a) and basicity (pK_b) constants were taken from Ref. 41.

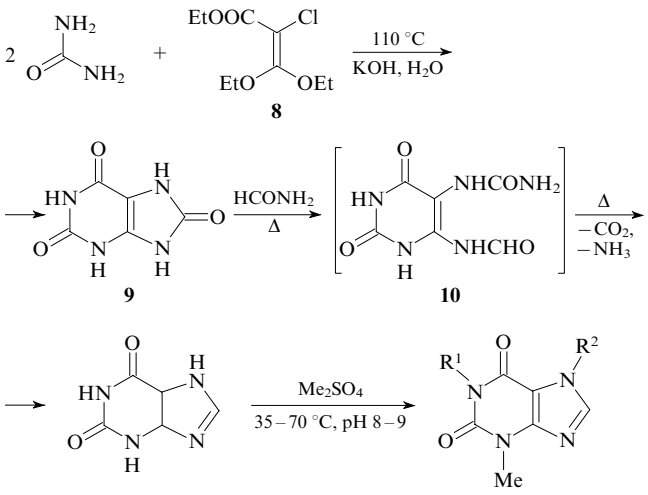
alkaloid from tea leaves and called it theine, and in 1838, Jobst and Mulder proved that theine was the same as caffeine. The caffeine synthesis was accomplished in 1897 by Fischer. Theobromine was isolated from the cocoa husks by Voskresensky in 1842. Later, this compound was also found in tea leaves. Tea also contains minor quantities of theophylline, which was discovered by Kossel in 1889. Pentoxyfylline was synthesized in 1965, and since 1972 a medicament based on this compound has been in clinical use in Germany under the brand name Trental.

More than sixty plants that contain caffeine and other methylxanthines in leaves or fruits are currently known. According to a review,[1] the highest caffeine content, up to 4.3%, is present in guarana seeds; up to 2.8% is present in tea leaves; up to 2.5% occurs in cocoa beans; up to 1.9% occurs in coffee beans; and up to 0.95% is present in the Paraguay tea (mate) leaves. The theobromine content in cocoa varies from 1.5% to 3%, and that in tea leaves is from 0.8% to 5.9%. The theophylline content in plants is the lowest: up to 0.013% in tea leaves and up to 0.02% in cocoa beans.

2. Preparation methods of methylxanthines

Wastes of tea industry (tea dust, cuts of leaves, *etc.*), coffee beans and other plants serve as the natural sources of caffeine, and cocoa beans are the sources of theobromine. Several methods are known for caffeine and theobromine recovery from natural raw materials. One of these is based on the counter-flow extraction of methylxanthines into boiling water. The aqueous extract is purified by precipitating the impurities by lead, calcium or magnesium salts. Then the filtrate is concentrated and the residue is recrystallized from cooled aqueous solutions.[43] In some cases, caffeine and theophylline are isolated from the aqueous extract by means of non-aqueous solvents (chloroform, dichloroethane, *etc.*).[43] A much higher yield of caffeine can be obtained by using the process developed in 1952 by Izmailov *et al.*,[44] according to which caffeine is recovered from aqueous extracts (even those with low caffeine contents) by adsorption on coal followed by desorption on treatment with chloroform or dichloroethane. Recently, supercritical carbon dioxide fluid extraction has been used more and more often to recover methylxanthines from natural sources. For example, for extracting caffeine from coffee beans, the beans are placed into an autoclave without preliminary grinding just in the bags, then CO_2 gas is fed to the autoclave, the pressure needed to generate the fluid is applied, and after some period of time, the supercritical carbon dioxide is poured into an open vessel. Under atmospheric pressure, CO_2 is gasified, and the extracted caffeine remains in a pure state.[45]

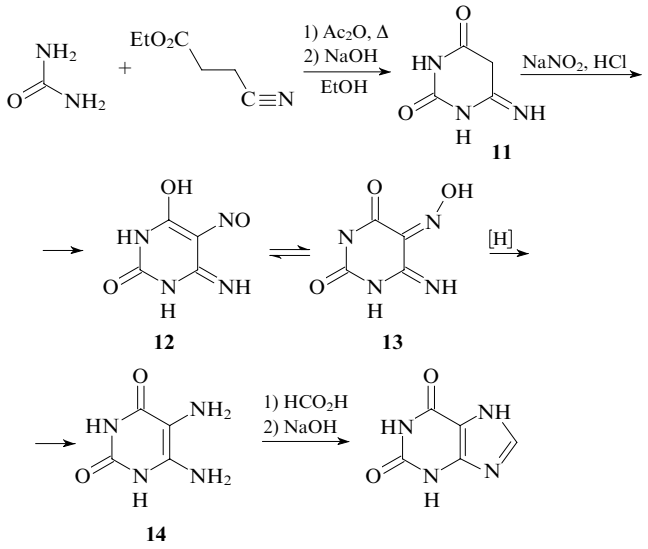
Several synthetic routes to methylxanthines have been described.[42,46] One route starts with thermal condensation of two urea molecules with ethyl 2-chloro-3,3-diethoxyacrylate (**8**) to give uric acid (**9**). On its heating with formamide, reduction (the addition of formamide) takes place to give intermediate **10**. After removal of carbon dioxide and ammonia, xanthine is left, which is subsequently methylated to give, depending on the reaction conditions, caffeine (pH 8.0–9.0, 35–70 °C), theobromine (KOH, MeOH, 60–70 °C) or theophylline (aqueous NaOH).



8 **9** **10**

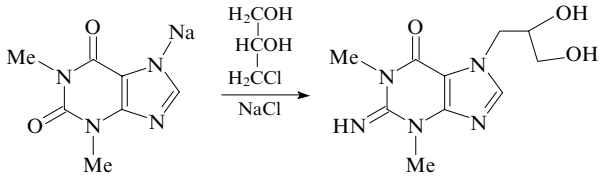
R^1, R^2 = H, Me

According to another synthetic scheme, the synthesis of xanthine is started with the condensation of urea with ethyl cyanoacetate in the presence of sodium ethoxide. The imine **11** thus formed is nitrosated and then nitroso derivative **12** is reduced *via* oxime tautomer **13** to diamine **14**. The latter is condensed with formic acid to give xanthine.



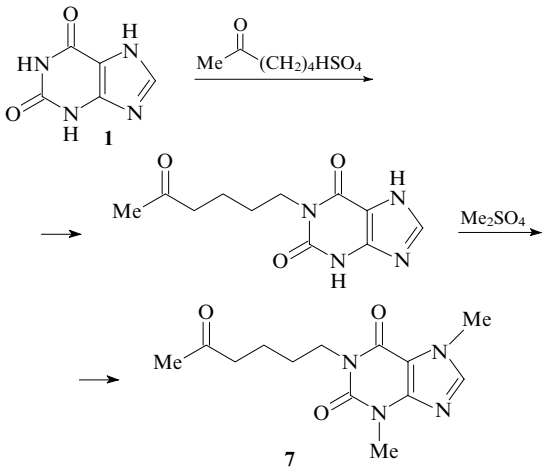
11 **12** **13** **14**

Diprophylline (**6**) is synthesized by the reaction of the sodium salt of theophylline with glycerol α-monochlorohydrin.[16]



6

Pentoxyphylline (**7**) is synthesized as follows:[16]



3. Biosynthesis of caffeine

One of the most interesting aspects of methylxanthine chemistry is the biosynthesis and metabolism in various plants: tea leaves and coffee and cocoa fruits.[1,47–60] Using labelled precursors, it was shown [48–50] that the principal biosynthetic route to caffeine in plants includes the following transformations:

xanthosine ⟶ 7-methylxanthonisine ⟶ 7-methylxanthine ⟶ theobromine ⟶ caffeine.

Various methyltransferases act as methylating agents, *S*-adenosylmethionine methyltransferase being most important among them.[49,58–60]

Apart from caffeine biosynthesis, processes of caffeine degradation (catabolism) take place in plants. According to a number of publications,[61–63] the key route of caffeine catabolism in plants can be represented as follows:

caffeine ⟶ theophylline ⟶ 3-methylxanthine ⟶ xanthine.

Xanthine undergoes further degradation according to the route typical of the purine catabolism being converted to carbon dioxide and ammonia *via* uric acid and allantoin.[64]

The functions of caffeine and other purine alkaloids in plants have not yet been adequately studied. Presumably, high concentrations of caffeine in juvenile tea leaves protect the plant from pathogenic microorganisms and insects.[65,66] It was found that spraying of plants with a 0.01%–1% solution of caffeine provides protection from slugs and snails.[66] The neurotoxic properties of caffeine were used in the design of transgenic tobacco able to produce caffeine and having enhanced stability against viruses and bacteria.[67,68] Furthermore, it was argued [69] that caffeine has allelopathic properties, *i.e.*, it can retard the development of microorganisms or plants.

4. Pharmacological properties

The biological activities of methylxanthines **2**–**7** are largely similar. They all excite the central nervous system, enhance the cardiac activity, reduce the bronchial muscle tone and stimulate the diuresis. Caffeine stimulates most strongly the central nervous system, while the action of theophylline and theobromine is weaker. Theophylline stimulates more efficiently the cardiac action and diuresis, while theobromine and especially caffeine have a less pronounced effect. Therefore, caffeine is used most often as a central nervous system stimulant, theophylline serves as a bronchodilator or diuretic agent, and theobromine is utilized to treat the cerebral vasospasm.[13–16,70–73] As regards the pharmacological properties, diprophylline resembles theophylline but is less toxic.[14] Pentoxyphylline resembles theobromine in its chemical structure; it has vasodilatory action and promotes the oxygen supply to tissues.[14] Currently, pentoxyphylline is a popular blood circulation enhancer agent used by sportsmen.

Despite the fact that methylxanthines have been clinically used for several decades, the mechanism of their action on the human organism is still not entirely clear. A certain role is presumably played by phosphodiesterase inhibition and accumulation of cyclic 3′,5′-adenosine monophosphate (cAMP) in tissues.[14] Theophylline is one of the strongest phosphodiesterase inhibitors. The accumulation of cAMP in the cells retards myosin binding to actin, thus reducing the contractive activity of smooth muscles and, in particular, relaxing bronchial spasms. The relaxation of muscles is also promoted by the ability of methylxanthines to depress the calcium ion transport through slow channels in the plasma membranes.

Of particular importance for the molecular mechanism of action of methylxanthines is their ability to block adenosine (purine) receptors.[74] Adenosine — an endogenous purine nucleoside, an adenine derivative — is considered as a natural ligand that specifically binds adenosine (purine) receptors, which occur in peripheral organs, cardiac muscle and bronchi. Methylxanthines function as exogenous ligands for adenosine receptors and block the action of adenosine. This is made possible by the structural similarity of adenosine and methylxanthines.

In the human or animal body, methylxanthines are metabolized, mainly in the liver. The key metabolic route of caffeine includes N-demethylation and oxidation to give paraxanthine (80%), theobromine (10%) and theophylline (4%) and smaller amounts of 1-methylxanthine, 7-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid and 1,3-methyluric acid.[21,24,70,75,76] The metabolism of theophylline affords mainly 1,3-dimethyluric (44.3%) and 1-methyluric (24%) acids and 3-methylxanthine (12.9%).[22,75,76] The theobromine metabolites are 3- and 7-methylxanthines and 7-methyluric acid. All methylxanthine metabolites are excreted with urine.

III. Methylxanthine isolation and preconcentration

Sample preparation is a necessary stage that precedes determination of methylxanthines in various objects. Extraction of methylxanthines from pharmaceutical agents and plant matrices, elimination of the adverse influence of accompanying components and decrease in detection limits — this is by no means the complete list of problems that are solved at the sample preparation stage. An important problem faced during the extraction of methylxanthines is related to their high hydrophilicity being manifested most often as low distribution ratios of these compounds. In addition, it is very important to isolate methylxanthines from various matrices without changing their nature; therefore, the selection and observance of sample preparation conditions are subject to stringent requirements. The most widely used methods for the recovery and preconcentration of methylxanthines include

extracting (liquid extraction from solid matrices) and liquid – liquid and solid-phase extraction.

1. Extracting

Extracting is used to recover methylxanthines from solid samples of some medicaments, tea, coffee, cocoa, *etc.* and is implemented in the following way: a weighed portion of the thoroughly ground solid sample (the optimal particle size depends on the analyzed material and varies from 0.5 to 8 mm) is placed into a vessel, the desired solvent is added, the mixture is stirred for a specified period of time (from several minutes to several hours), and then the phases are separated by filtration.

When methylxanthines are determined in medicaments, the sample preparation includes most often sample grinding and dissolution in water,[77–90] in aqueous sulfuric acid,[91] or in acetate [92] or borate [93] buffer. The other suitable solvents include ethyl acetate,[94] methanol,[95] aqueous methanol,[96, 97] aqueous acetonitrile [98] or aqueous ethanol.[99–102] For intensification of the extraction, the dissolution is performed in some cases in an ultrasonic bath.[79, 80, 99]

Extracting of caffeine and other methylxanthines from tea is performed most often using hot water as the solvent.[103–115] A weighed tea sample is covered by boiling water and mashed for the specified period of time. The conditions for recovery of methylxanthines from tea by hot water described in the literature have not still been unified and differ both in the sample weight (0.1 to 1.5 g) and the volume of water (from 20 to 100 ml) and in the mashing conditions (temperature, time, *etc.*). The key interfering components, catechols, which account for up to 30% of the weight of dry green tea leaves,[116] are precipitated in some cases by lead acetate.[108–111] Apart from water, the extraction of methylxanthines from tea may be accomplished by ethanol,[117] aqueous methanol,[118–120] aqueous acetonitrile [121, 122] and acids.[123] During the preparation of tea samples, double preconcentration is sometimes used: the first stage is liquid extraction from solid matrices, and this is followed by liquid – liquid [123] or solid-phase extraction.[119]

The extraction of methylxanthines from coffee also makes use of hot water,[106, 109, 113, 124–131] buffer solution,[132] ethanol [107] or methanol.[126] The samples are additionally purified by extraction with chloroform [130] or dichloromethane [124, 128] or by solid-phase extraction using cartridges filled with silicas chemically modified by octadecyl groups.[106, 127]

Cocoa is first defatted by treatment with dichloromethane [133] or petroleum ether,[134, 135] and after that, methylxanthines are extracted into hot water [134, 135] or a methanol – water mixture.[133]

Methylxanthines are extracted from chocolate into a methanol – water mixture,[136] chloroform [79] or hot water.[135, 137] In the last-mentioned case, chocolate is preliminarily defatted by petroleum ether [135] or hexane.[137] Examples of extractants for liquid extraction of methylxanthines from other solid matrices are given in Table 3.

2. Liquid – liquid extraction

Liquid – liquid extraction is a traditional preconcentration process, which is often used as one of the sample preparation stages to recover methylxanthines from beverages,[144, 145] biological fluids (urine,[20, 22, 146–151] blood serum and plasma,[82, 88, 152–158] saliva,[157, 158] sweat [159]) and complex drug formulations.[160]

The liquid – liquid extraction of methylxanthines is performed using chloroform,[145, 152] dichloromethane,[157] ethyl acetate [88, 146, 156] and isopropyl alcohol.[158] In order to increase the distribution ratios, two-component extraction mixtures of organic solvents are often used in practice; most often, these mixtures contain various ratios of chloroform and isopropyl alcohol.[144, 154, 159] For example,[154] theophylline and acetaminophen (internal standard) were extracted from blood plasma with a chloroform – isopropyl alcohol mixture (75 : 25). The extract was analyzed by thin-layer

Table 3. Extractants used to recover caffeine and other methylxanthines and extraction conditions.

Specimen	Methylxanthine	Extractant (extraction conditions)	Method of analysis	Ref.
Mate leaves	caffeine, theobromine	EtOH (heating on a water bath)	GC	5
Citrus blossoms	caffeine	HCl (USB; SPE on Kieselgur column, hexane flow-through, desorption with CH_2Cl_2, evaporation, dissolution in water)	HPLC-UV	6
Cola nuts	"	acetone (twofold extraction)	HPLC-UV	7
BAS	"	EtOH – H_2O (USB)	CZE	102
Coffee	"	H_2O (SPE on a Sep-Pak C_{18} column, desorption with water – methanol)	HPLC-UV	128
Japanese fungus	"	$CHCl_3$ – MeOH	GC-MS	138
Hair	"	$CHCl_3$	GC-MS	139
Soil	"	CH_2Cl_2, CH_2Cl_2 – Me_2CO, Me_2CO (extraction, evaporation, dissolution in hexane)	GC-MS	140
Tobacco	"	EtOAc [soaking in a citrate buffer solution (pH 4.5), filtering, extraction with ethyl acetate, reextraction with HCl]	GC-MS	141
Standard samples of BAS	"	H_2O – MeOH (USB); MeOH (USB)	HPLC-MS HPLC-UV	142
Sludge from treatment plants	pentoxyphylline, caffeine, *etc.*	MeOH, Me_2CO (successive extraction, USB; evaporation, dissolution in H_2O, SPE on an RP-C18 column, desorption with MeOH, evaporation, dissolution in a phosphate buffer solution)	HPLC-MS	143

Notes. The following abbreviations are used: SPE is solid-phase extraction; BAS is biologically active supplement; USB is ultrasonic bath; GC is gas chromatography; HPLC is high-performance liquid chromatography; CZE is capillary zone electrophoresis; UV and MS are ultraviolet and mass-spectrometric detectors.

chromatography on silica gel-based plates and a mixture of acetic acid, isopropyl alcohol and toluene (1 : 12 : 6) as the mobile phase. A chloroform – isopropyl alcohol mixture was used to extract caffeine and some its metabolites from urine prior to their determination by high-performance liquid chromatography.[147–149] In addition, dichloromethane – methanol,[20] ethyl acetate – isopropyl alcohol[23] and dichloromethane – isopropyl alcohol[153] mixtures were utilized. For example,[150,151] for a HPLC determination of caffeine in urine, it was extracted during the sample preparation stage with a mixture of dichloromethane with methanol (9 : 1). A diethyl ether – dichloromethane – isopropyl alcohol ternary mixture (6 : 4 : 1) was employed to extract theophylline from blood plasma prior to its determination by high-performance thin-layer chromatography.[155]

Quantitative characteristics of the extraction of caffeine, theophylline and theobromine with various solvents and their mixtures were reported.[161,162] Upon analysis of the distribution ratios (D) in systems water (pH 2–7) – organic solvent (chloroform, dichloromethane, 1,2-dichloroethane, isobutyl and isoamyl alcohols and ethyl and amyl acetates), it was shown[161] that caffeine is extracted most efficiently with chloroform (D = 17.76), theophylline is best extracted with isoamyl alcohol (D = 1.46) and theobromine is best extracted with isobutyl alcohol (D = 0.57).

3. Supercritical fluid extraction

Supercritical fluid extraction is used to recover caffeine from tea,[163–167] mate crushed leaves and young shoots,[168–171] guarana seeds[171–173] and coffee beans;[174] and to recover caffeine and theobromine from cocoa beans.[171,175–177] Using this method, it is possible to increase considerably the efficiency of extraction of methylxanthines from plant specimens, accelerate sample preparation process and eliminate the use of toxic organic solvents.

As a rule, supercritical carbon dioxide is used. Since the polarity of CO_2 is relatively low, polar compounds are often added; for this purpose, water,[164] ethanol,[171,178,179] isopropyl alcohol[178] and methanol[180] have been studied. Using the supercritical fluid extraction of caffeine from green tea leaves, it was demonstrated that the degree of extraction increases with increase in the temperature, pressure and water content in supercritical CO_2 and reaches a maximum (66%) at 323 K, 40 MPa and 20.8% of water.[164] The solubility of caffeine was found to approximately double as the ethanol content in the supercritical CO_2 increases from 5% to 10%.[178] The solubilities of theophylline in organic solvents and in supercritical CO_2 in the absence and in the presence of methanol[181] and ethanol[182] were compared. It was demonstrated that caffeine and theobromine contained in cocoa are extracted more efficiently into supercritical ethane than into supercritical CO_2, which is related to the coextraction of the cocoa oil, which is better soluble in the former fluid.[177]

The supercritical fluid extraction is used not only for recovery of caffeine and theobromine from various plant products and for decaffeination of coffee and tea but in analytical chemistry as a modern sample preparation technique for the determination of these compounds in various objects. A procedure for the supercritical fluid extraction of methylxanthines and other biologically active compounds present in mate leaves followed by analysis of fractions by gas chromatography/mass spectrometry made it possible to identify 51 compounds (Fig. 1).[169] Caffeine in coffee oil was identified by HPLC, the oil being recovered from green coffee beans by supercritical fluid extraction.[174]

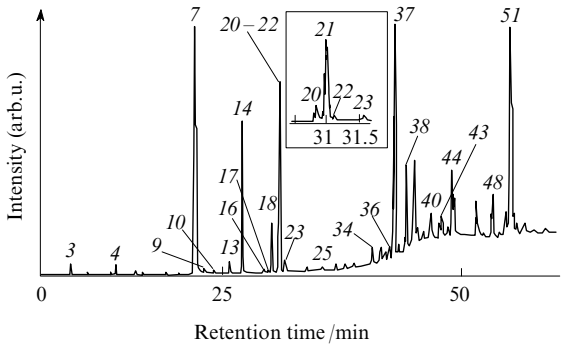


Figure 1. Chromatogram of the extract of mate leaves obtained by supercritical fluid extraction (the numbers of very small peaks are omitted). The numbers of larger peaks are given; the peak identification and the extraction and analysis conditions were reported.[169]

4. Solid-phase extraction and other sorption methods

The solid-phase extraction technique is used more and more widely to recover and preconcentrate caffeine and other methylxanthines and their metabolites from blood,[27,152,183–186] urine[28,187–190] and breast milk[191] prior to chromatographic determination. The method is based on the distribution of the trace component to be isolated between the mobile and stationary phases as a result of sorption processes taking place in a small column (cartridge). In addition, the solid-phase extraction is used to recover caffeine from energy beverages,[144,187,192,193] coffee,[106] cocoa,[106,135] waters,[34,36,194–204] in particular, in combination with preconcentration methods such as liquid extraction from solid matrices and liquid – liquid extraction. In some cases, solid-phase extraction is used to remove the interfering components contained in the analyzed objects. For example, for the determination of caffeine in tea, polyphenol derivatives were removed using polyvinylpyrrolidone-filled cartridges.[105,205–207]

The recovery and preconcentration of methylxanthines are performed most often using silica gels modified by hydrophobic alkyl groups (mainly C_{18}) (Refs 28, 106, 189–192, 194–196). The cartridges Sep-Pac C_{18} (Refs 27, 135) and HySphere-C_{18} are commercially manufactured and widely used.[184] Cartridges filled by polymeric sorbents: Oasis HLB,[28,183,195,197–202] Abselut Nexus,[28] StrataX,[203,204] LiChrolut EN,[34] HySphere Resin GP,[184] polystyrene – divinylbenzene[36,184,201,203,208] and polymethacrylate[188,209] sorbents have found use. A comparison of the efficiency of methylxanthine extraction using different cartridges was reported.[184,196,201,203] The cartridges HySphere Resin GP,[184] Sep-Pac C_{18},[196] Oasis HLB[201] and Strata-X,[203] which combine high sorption capacity and desorption efficiency, were acknowledged to be the best.

For the selective solid-phase extraction of methylxanthines, molecularly imprinted materials have been proposed: polymers with molecular imprinting of caffeine[187,193,210–214] and theophylline;[152,185,214–221] sol – gel imprinted silicas;[222,223] and materials based on

cellulose,[224–226] silica gel[186,216] and carbon nanotubes.[227–229] The essence of the molecular imprinting is the preparation of organic or inorganic materials whose structure has sites (imprints) capable of complementary interactions with molecules used as templates in the synthesis of these materials.[230–233] The molecularly imprinted materials offer some advantages over traditional sorbents for solid-phase extraction, specifically, high selectivity, relative simplicity of the synthesis, rather low cost, stability in various media and compatibility with organic solvents.

Sample preparation using solid-phase extraction comprises a number of stages: activation and conditioning of the cartridge by washing with a solvent, passing-through of the sample, washing of the cartridge to remove the remainder of the test solution and elution of the target components. Most often, methylxanthines are eluted with methanol[27,187,188,190,191,198] or aqueous methanol solutions.[28,185,187,193] Also, other organic solvents are used: ethyl acetate,[183,197,199] chloroform,[135] dichloromethane,[189] acetonitrile,[193] acetone[194] or a chloroform–isopropyl alcohol mixture.[144]

In a number of studies,[145,234,235] it was proposed to use solid-phase microextraction, which is based on distribution of the analyte between the liquid phase and the sorbent layer supported on a quartz or glass rod, most often, on a microsyringe needle for gas chromatography, to preconcentrate methylxanthines prior to gas chromatography/mass spectrometry determination. Microfibres based on silicon-containing substrates coated by various polymers are used as sorbents in this method. The solid-phase microextraction method was utilized to extract caffeine from energy beverages, tea and coffee[145] and to extract caffeine, theophylline and theobromine from biological fluids.[234,235]

A new version of sorption preconcentration was employed[236] to extract caffeine from blood plasma. Glass magnetic stirrer bars coated by a restricted access material, namely, alkyldiol silica, served as the sorbent in this study. This technique allows direct extraction of analytes from biological fluids, because the coating used prevents the macromolecules of proteins and other biological fluid components from penetrating the material layer and being adsorbed on the surface. The HPLC detection limit for caffeine after using this sorption preconcentration procedure was 0.025 μg ml^{-1}.

IV. Methylxanthine determination methods

As noted above, methylxanthines are determined in objects that differ in composition and complexity, and the requirements to the analytical methods are also substantially different. Relatively large amounts of methylxanthines are present in plants, food products, beverages and medicaments but during the sample preparation, which is performed most often by liquid extraction from solid matrices, catechols, polyphenols and other biologically active compounds that markedly affect determination also pass to the aqueous or aqueous-organic extracts. Conversely, biological fluids and natural waters not only have complex compositions but also contain only small amounts of these compounds; analysis is performed after preconcentration, for example, by solid-phase extraction. Methods that have found wide use for determination of methylxanthines include various versions of chromatographic and electrophoretic techniques, UV and IR spectroscopy. Much fewer publications are devoted to determination of methylxanthines by electrochemical methods.

1. Determination in plants, food products and beverages

The existing procedures for methylxanthine determination in plants, food products and beverages are characterized in Table 4. Reversed-phase HPLC is used most often for separation, identification and determination of methylxanthines in tea and mate leaves, guarana seeds, coffee beans, cocoa beans, chocolate and energy beverages. In tea and mate leaves and in cocoa beans, caffeine and theobromine are determined most often and theophylline, which is present in much lower quantities, is determined more rarely. In coffee, caffeine is determined in most cases and theobromine is seldom determined; determination of the latter is hampered by its low content with respect to the caffeine content (the theobromine : caffeine ratio is up to 1 : 1000). In energy beverages, caffeine is determined.

The stationary phases used in reversed-phase HPLC include various hydrophobic sorbents with grafted alkyl groups, as a rule C_{18}, more rarely, C_{16} or C_{12}. In most cases, columns packed by 3–5 μm sorbent particles are used. Some types of commercially available stationary phases used to determine methylxanthines are presented in Table 5.

Monolithic columns are advantageous from the hydrodynamic standpoint.[129,237] By increasing the eluent flow rate, decreasing the washing and equilibrating times, decreasing the signal-to-noise ratio, researchers[129] succeeded in separating caffeine, theophylline and theobromine on a Chromolith SpeedRod RP-18e monolithic column in 0.68 min. The caffeine detection limit was 0.1 μg ml^{-1}. An HPLC separation and determination of methylxanthines was performed on monolithic molecularly imprinted columns with caffeine or theophylline imprints.[246–250]

The most common mobile phases include methanol or acetonitrile mixtures with an acetate or phosphate buffer solutions and aqueous–organic mixtures with various contents of acetic, phosphoric or formic acid (see Table 5). Gradient elution can ensure the separation of complex mixtures of methylxanthines, catechols and other polyphenols and shorten the analysis time. By using gradient elution,[112] it was possible to separate caffeine, theobromine, theophylline and six catechols in 17 min after their extraction with hot water from tea leaves.

When methylxanthines are determined by reversed-phase HPLC, spectrophotometric UV detectors are often used, the detection being usually performed at 270–280 nm. In some cases, diode-matrix[104,120,122] or IR detectors[242] serve for methylxanthine determination. The problems faced by the use of amperometric detectors are mainly due to the difficulty of oxidation of these compounds at the working electrode (1 V for theophylline and 1.4 V for theobromine and caffeine).[106] The HPLC detection limits of methylxanthine vary from 0.01 to 0.2 μg ml^{-1} (see several papers[129,209,270,282]).

In recent years, HPLC with mass spectrometric detection has been used more and more often to identify and determine methylxanthines and other compounds present in beverages and foodstuffs.[117,125,243–245] This method was used[125] to analyze coffee samples for caffeine, trigonelline, nicotinic acid and saccharose. The determination included treatment of coffee with hot water, removal of catechols by lead acetate, filtering and chromatographic separation of compounds on a Spherisorb S5 ODS2 column with gradient

Table 4. Methods used to determine methylxanthines in various objects.

Specimen	Analytes	Method of analysis	Ref.	Specimen	Analytes	Method of analysis	Ref.
Tea leaves	**2**, **3**, **5**, catechols	HPLC	18, 83, 103, 104, 105 – 107, 110 – 114, 117, 119 – 122, 133, 163 – 167, 205 – 207, 209, 237 – 252	mate leaves	**2**, **5**, phenolic compounds	HPLC	123, 171, 275
					5, *etc.*	GC-MS	168 – 170
				guarana seeds	**2**, **5**	HPLC	171
	2, **3**, **5**	CZE	93, 113, 253				
	5, *etc.*	MEKC	254, 255	cocoa chocolate	**2**, **5**	HPLC	133 – 137, 171, 177, 237, 276
	2, **5**	SP	108, 109, 256		**2**, **5**	CZE	79
	2, **3**, **5**	FIA	206, 257		**2**, **5**	SP	277, 278
	2, **3**, **5**	SSP	132		**2**	RS	279
	2, **5**, polyphenols	IRS	118, 258 – 261	energy beverages	**5**, *etc.*	HPLC	106, 129, 133, 187, 193, 210, 237, 280
	5	VA	262 – 264		**5**	HPTC	281
	5	S	209, 265, 266		**3**,**5**	CEC	282
		HPTC	115		**5**	GC-MS	145, 283
Coffee beans	**2**, **3**, **5**, *etc.*	HPLC	11, 18, 83, 106, 109, 124 – 127, 129, 131, 133, 174, 187, 237, 267 – 271		**5**	FIA	32, 257, 284
					5	SP	257
	5, *etc.*	MEKC	272		**5**	RS	192
	2, **3**, **5**	IRS	261, 273, 274		**5**	MS	285 – 287
	2, **5**	SP	109, 124		**5**	VA	264, 288, 289
	5	VA	128		**5**	ABS	290, 291

Notes. The following abbreviations are used: MEKC is micellar electrokinetic chromatography; SF is spectrophotometry; FIA is flow-injection analysis; SSP is solid-state phosphorescence; IRS is infrared spectroscopy; VA is voltammetry; S is sensor; HPTC is high-performance thin layer chromatography; GC-MS is gas chromatography/mass spectrometry; CEC is capillary electrokinetic chromatography; RS is Raman spectroscopy; MS is mass spectrometry; ABS is amperometric biosensor.

Table 5. Examples of determination of methylxanthines by reversed-phase HPLC.

Specimen	Column	Mobile phase	Detector (wavelength)	Ref.
Tea leaves	Mightysil RP-18	MeCN – H_2O (see [a]), MeCN – H_2O – H_3PO_4 (see [b])	UV (231 nm)	104
	Develosil ODS-HG	MeCN – H_2O – H_3PO_4	UV (231 nm)	122
	GL-Sciences Inertsil ODS-3	MeOH – H_2O – AcOH	UV (272 nm)	206
	Phenomenex RP-MAX C_{12}	MeCN (see [a]), HCOOH – H_2O (see [b])	MS	244
Tea leaves, coffee beans	LiChrospher 100 RP-18	EtOH – MeCN – H_2O	UV (273 nm)	18
Tea leaves, coffee beans, cocoa beans, beverages	RP-C_{18}	MeOH – H_2O (pH 3.5)	amperemetre	106
Tea leaves, coffee beans, beverages	FastGradient Chromolith	MeCN – H_2O	UV (274 nm)	237
Mate leaves	Supelcosil LC-18	AcOH – H_2O (see [a]), MeOH (see[b])	UV (265 nm)	123
Coffee beans	LiChrospher 100 RP-18	H_2O – HCOOH (see [a]), MeCN – H_2O – HCOOH (see [b])	UV (280 nm)	127
	Kovasil MS-C_{18}	MeCN – H_2O – AcOH	UV (270 nm)	269
	Aquasil C_{18}	MeCN – H_2O (pH 4.3)	UV (270 nm)	270
Cocoa beans	Nova-Pak C_{18}	MeOH – H_2O	UV (274 nm)	134
	Supelcosil LC-18-DB	MeOH – MeCN – H_2O	UV (275 nm)	276
Chocolate	Hypersil ODS	MeOH – H_2O – HCOOH	UV (280 nm)	136
	Zorbax C_{18}	MeCN – H_2O (pH 2.5)	UV (274 nm)	137
Beverages	Zorbax Eclipse XDB-C_8	MeCN – H_2O (pH 8.0)	UV (273 nm)	280
Dietetic product	Phenomenex Luna C_{18}	MeOH – H_2O – AcOH	MS	144

[a] Phase A. [b] Phase B.

elution with a mixture of 0.3% aqueous formic acid and methanol. The detection limit of caffeine was 11.9 ng ml^{-1}.

Among other chromatographic methods used to determine methylxanthines in plants, foodstuffs and beverages, noteworthy are ion HPLC,[83, 251, 252] high-performance thin layer chromatography [115, 281] and gas chromatography/mass spectrometry.[145, 169, 283] Ion HPLC was used [83] to determine caffeine, theobromine and theophylline in tea, coffee and beverages. The Dionex HPIC-CS3 cation exchange resin and the Dionex OmniPac PAX-100 anion exchange resin were used as stationary phases, while 0.1 M HCl and a 0.015 M KOH + 1% acetonitrile mixture served as the

mobile phases. The detection limits of caffeine, theophylline and theobromine were 0.1, 0.06 and 0.03 μg ml^{-1}, respectively. A procedure for caffeine determination in tea leaves by high-performance thin layer chromatography with a densimetric detector was developed.[115] The authors demonstrated that using selective complexation of catechols with iron(III) cations, it is possible to eliminate their interfering effect in the analysis of various green and black tea brands. The caffeine detection limit was 100 ng. High-performance thin layer chromatography with UV detection served to determine caffeine in beverages.[281] Silica gel was used as the stationary phase and a chloroform–ethanol–acetic acid–acetone–water mixture (54 : 27 : 10 : 2 : 2) was the mobile phase.

Capillary zone electrophoresis determination of methylxanthines is usually carried out in alkaline solutions.[79, 93, 113, 253, 272] In most cases, a borate buffer solution is used as the supporting electrolyte. The detection is accomplished by UV-spectrophotometric[79, 113, 253] or amperometric[93] detectors. A mixture of caffeine, diphyllin, theobromine, enprophylline (3-propylxanthine) and theophylline was separated[79] in 2 min (the compounds are listed in the order of elution) (Fig. 2). Using capillary zone electrophoresis, caffeine, theophylline and theobromine were determined in tea, coffee and coca-cola.[113]

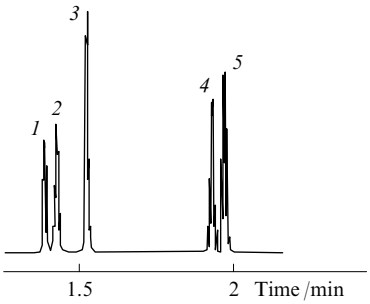


Figure 2. Electrophoregramme of a mixture of compounds containing caffeine (*1*), dyphylline (*2*), theobromine (*3*), enprophylline (*4*) and theophylline (*5*). The conditions of analysis were reported.[79]

Micellar capillary electrokinetic chromatography with UV detection was used to determine caffeine, catechols and other compounds in tea,[254, 255] coffee[272] and other beverages.[282] A mixture of caffeine, theanine, gallic acid and ascorbic acid and a mixture of five catechols were separated[254] in 13 min on a non-modified quartz capillary (57 cm long). The detection limits were 1–20 μg ml^{-1}.

A number of publications deal with determination of methylxanthines by spectrometric methods. Caffeine, theobromine and polyphenols in tea leaves were determined by IR spectroscopy with multidimensional calibration.[118, 258–260] Fourier transform IR spectroscopy was used to quantify caffeine in tea leaves[261] and coffee beans[261, 273, 274] after its extraction into chloroform. The measurements were carried out at 1659 cm^{-1}. The limit of detection was 1–3 μg ml^{-1}. Theobromine in cocoa beans was identified by Raman spectroscopy.[279] The solid-state FT Raman spectroscopy was used to determine caffeine in energy beverages; first, caffeine was extracted by a solid-phase extraction procedure.[192] For identification and determination of caffeine in beverages, mass spectrometry was used.[285–287] It was noted[132] that caffeine, theophylline and theobromine can be determined in tea, coffee and chocolate by a solid-state phosphorescence technique at room temperature with detection limits of 0.78–1.8 ng. Spectrophotometric determination of methylxanthines in tea, coffee and beverages is also performed using various chemometric approaches.[108, 109, 124, 256, 277, 278]

Among electrochemical methods, square-wave,[128, 262, 289] cyclic[263, 288] and differential pulse[264] voltammetry are used for caffeine determination in such objects. Most often, this is done with chemically modified graphite electrodes on which caffeine is oxidized at 1.4–1.5 V. The limits of detection of caffeine by electrochemical methods were 9.2 μg ml^{-1} (Ref. 262), 0.3×10^{-8} (Ref. 128), 1.5×10^{-8} (Ref. 288) and 1.5×10^{-8} mol litre^{-1} (Ref. 264). Piezoelectric quartz sensors coated with caffeine-imprinted polymer were proposed for caffeine determination in tea and coffee.[209, 265, 266] Biosensors for detecting caffeine in beverages have been developed.[290, 291]

V. Determination in medicaments

Pharmaceutical analysis is often performed by means of spectrophotometric methods, which are included in International Pharmacopoeia and national pharmacopoeias of many countries. For verification of the authenticity of drugs, for monitoring of production processes and in some other cases, UV spectrophotometry is used. Determination procedures of caffeine[85, 86, 90, 91, 96, 97, 99, 100, 292–299] and theophylline[300, 301] in various pharmaceuticals based on the intrinsic absorption of solutions in the UV region have been reported. Different versions of mathematical treatment of the spectra (derivative spectrophotometry, Firordt method, multivariant regression analysis and other) are used to increase the accuracy of determination of methylxanthines in the presence of other compounds that are components of the same medicaments. In some publications, caffeine and other drug substances are determined in the flow injection analysis mode with UV detection.[302–306]

The selectivity of determination of methylxanthines can be enhanced by analyzing their intensely coloured derivatives in the visible spectral range. A method for determination of caffeine and theophylline based on oxidation of methylxanthines with sodium periodate in the presence of acetic acid has been described.[95] The oxidation products react with 3-methylbenzotriazol-2-yl hydrazone hydrochloride to give blue-coloured compounds with absorption maximum at 630 nm. 4-Nitrophenyldiazonium tetrafluoroborate was proposed as a reagent for spectrophotometric determination of caffeine and theophylline in medicaments. This reagent is azo-coupled with the products of alkaline hydrolysis of caffeine and theophylline yielding coloured compounds.[307] A kinetic spectrophotometric method for determination of theophylline, dyphylline and proxyphylline in the drug Novofilin Retard after converting these compounds to coloured azo derivatives by the reaction with diazotized sulfanilic acid has been developed.[81]

Infrared spectroscopy is also considered as a pharmacopoeial analytical method; it is used not only to establish the authenticity of medicaments based on the standard pharmacopoeia spectra but also for quality control of dosage forms. IR spectroscopy was used to determine caffeine in tablets.[308, 309] The IR spectra of methylxanthines were

reported.[310] Low-temperature plasma mass spectrometry was proposed for fast screening of active components of drugs, including those containing caffeine.[311]

Chromatographic methods are invoked for analysis of multicomponent pharmaceuticals. Most often, caffeine and theophylline present in various pharmaceutical compositions are separated and determined by reversed-phase HPLC with UV [82, 237, 312–318] or diode array [98, 319–322] detection. Mixtures of drug substances with markedly different chromatographic properties are separated using a gradient elution mode.[98, 319–322] In the gradient mode, caffeine, paracetamol, propyphenazone, phenobarbital and drotaverine present in the drug 'Pentalgin with propyphenazone and drotaverine' are separated in 10 min.[98] Ion chromatography was utilized to determine caffeine and theophylline in tablets and injection preparations [83] and to determine caffeine, theophylline and theobromine in pharmaceuticals.[251] Theophylline in tablets was quantified by micellar liquid chromatography with luminescence detection.[94] A normal-phase chromatography procedure (Zorbax CN column) was used, an ethyl acetate–hexane mixture (6 : 4 or 1 : 1) containing 0.01 mol litre^{-1} of Tb^{III} chloride served as the mobile phase. The detection limit was 9 ng ml^{-1}.

In some publications, reversed-phase high-performance thin layer chromatography was used to determine caffeine in medicaments.[101, 323, 324] For example, caffeine and paracetamol were separated on RP-TLC-18W plates using a methanol–glacial acetic acid–water mixture as the mobile phase.[101] By high-performance thin layer chromatography, theophylline and etophylline were determined.[325]

Capillary zone electrophoresis [79, 93, 102, 326–330] and micellar electrokinetic chromatography [77, 78, 87, 88, 328–330] are started to be widely used for the determination of caffeine and other methylxanthines in pharmaceuticals. The advantages of this method are high separation efficiency and rapidity and simplicity of analysis (Table 6).

The electrochemical methods of methylxanthine determination are based on the oxidation of these compounds on various electrodes. The theophylline oxidation on a carbon electrode was found to involve π-bond cleavage in the conjugation chain of this molecule and to be facilitated in the presence of trimethylcetylammonium bromide.[80] Differential pulse voltammetry is suitable for determining this compound in medicaments to a detection limit of 1.85×10^{-7} mol litre^{-1} (Ref. 80). For sensitive determination of theophylline, electrodes based on carbon modified with cobalt phthalocyanine nanoparticles [331] and glassy carbon electrodes modified with multi-walled carbon nanotubes have been developed.[332, 333] Using a boron-doped diamond electrode, square-wave voltammetric determination of caffeine and paracetamol in tablets was carried out.[92] A chemically modified electrode was used to determine caffeine by square-wave voltammetry.[334] For determination of pentoxyphylline in dosage forms, a glassy carbon-based electrode was used.[335]

A potentiometric sensor with a poly(vinyl chloride) membrane containing dibutyl phthalate, 4-(2,5-dimethoxyphenyl)-2,6-diphenylthiopyrylium perchlorate and oleic acid was developed for determination of theophylline.[84] By combining the lipophilic tetrakis[3,5-bis(2-methoxyhexafluoroprop-2-yl)phenyl]borate cation exchanger and 2-nitro-2′-fluorodiphenyl ether, a caffeine-selective membrane electrode was designed suitable for determination of this compound in various pharmaceuticals.[336]

1. Determination in biological objects

Determination of methylxanthines in biological objects is a challenge for the analytical chemistry of this class of compounds. This is due to not only small amounts of specimens and low contents of the analytes but also to the complex multicomponent composition of biological fluids, which contain chemically different inorganic and organic compounds: trace elements, amino acids, polypeptides, proteins, enzymes and so on. Even a relatively simple biological fluid such as urine is known to contain several hundreds of organic compounds. All this imposes certain restrictions on the range of suitable methods of sample preparation from biological materials and on their compatibility with the subsequent determination methods. Most often, methylxanthines are isolated from biological objects by liquid–liquid or solid-phase extraction (see above) after precipitation of proteins. Table 7 summarizes the general knowledge about the determination of methylxanthines in human blood plasma and serum, saliva, breast milk, sweat and hair and in animal urine and tissues.

Analysis of biological objects for methylxanthines is needed for several reasons. First, this is necessary for estimating the efficiency of therapy by methylxanthine-containing drugs. It is especially important to monitor the blood plasma level of theophylline, which has a very narrow therapeutic and toxic concentration ranges (Refs 152–155, 158, 183–185, 332, 337, 338, 340–344, 347–356). Often,

Table 6. Examples of determination of caffeine and other methylxanthines by capillary zone electrophoresis and micellar electrokinetic chromatography.

Analytes	Method of analysis	Buffer solution	Detector (wavelength)	Time of analysis /min	Ref.
2, **3**, **5**	MEKC	phosphate buffer solution (pH 8.5) + SDS	ECD	13	77
5, paracetamol, propyphenazone	MEKC	borate buffer solution (pH 9.0) + SDS	UV (200 nm)	10	78
2, **3**, **5**, dyphylline, enprophylline	CZE	borate buffer solution (pH 9.4)	UV (190 nm)	2	79
2, **3**, dyphylline	MEKC	borate buffer solution (pH 9.0) + SDS	UV (203 nm)	4	87
2, **3**, **4**, **5**, 1,3,7-trimethyluric acid	MEKC	phosphate buffer solution (pH 11.0) + SDS	UV (274 nm)	2	88
2, **3**, **5** and their metabolites	CZE	carbonate buffer solution (pH 10.0)	UV (254 nm)	16	326
5, aminacetophen, butalbital	MEKC	phosphate buffer solution (pH 9.0) + SDS	UV (220 nm)	12	328

Note. The following abbreviations are used: MEKC is micellar electrokinetic chromatography, SDS is sodium dodecyl sulfate, ECD is electrochemical detector; CZE is capillary zone electrophoresis.

Table 7. Methods used for determination of methylxanthines in biological specimens.

Specimen	Analytes	Method of analysis	Ref.
Human blood plasma	**2**, **3**, **5**, **7** and their metabolites	HPLC	18, 22, 27, 82, 156, 158, 184, 189, 236, 337 – 339
	3, **5**, **7**	CZE	340
	5	GC	157
	3	HPTC	154, 155, 341
	3	SF	153
Human blood serum	**2**, **3**, **5** and their metabolites	HPLC	28, 152, 184 – 186, 235, 342 – 346
	3, **5**	MEKC	347
	3, **5**	SF	17, 96, 348, 349
	3, **5**	NMR	350, 351
	3, **5**	S	352 – 357
	3	GC	183
Human whole blood	**2**, **3**, **4**, **5**	GC	234
Human urine	**2**, **3**, **5** and their metabolites	HPLC	18, 20, 22, 25, 28, 29, 146 – 151, 187 – 189, 312, 343, 345, 358, 359
	2, **3**, **4**, **5**	GC	234, 360, 361
	2, **3**, **5**	MEKC	362
	3, **5**	CZE	363
	5	S	352
	3	VA	80
Human saliva	**3**	HPLC	158
	5	GC	157
Human breast milk	**2**, **3**, **4**, **5**	HPLC	191
Human sweat	**5**	GC-MS	159
Human gastric contents	**5**	S	364
Human nails	**5**	GC-MS	365
Human hair	**5**	GC-MS	139
Animal urine and tissues	**5**, **7**, **6**	HPLC	26, 366
	3, **5** and their metabolites	CZE	88, 190

blood and urine tests for identification and quantification of caffeine and its metabolites are carried out.[157, 188, 338, 342 – 344, 350 – 352] Caffeine and its natural metabolites — theophylline and theobromine — get into a human body with food, drinks and drugs in relatively large amounts.[200] On average, every human in the world takes daily up to 829 mg of caffeine, and the caffeine concentration in blood plasma varies from 5 to 20 μmol litre^{-1} (Ref. 367). The content of these compounds in a human body is fairly high: about 2.5 mg per kg of body weight. Currently, caffeine is classified as a stimulant the use of which is permitted but the International Olympic Committee Medical Commission establishes the maximum allowed caffeine concentration in the urine of sportsmen to be 12 μg ml^{-1}, which implies the need to analyze urine for caffeine and caffeine metabolites.[19, 20, 146, 149 – 151, 360, 362] The doping control also applies to sport animals (in particular, horses) in whose blood the presence of caffeine, theobromine or theophylline is totally forbidden.[18] The contents of caffeine and caffeine metabolites in biological fluids can be used to estimate the activity of cytochrome P450 (CYP 450) isoenzymes, which catalyze the oxidative metabolism of various chemical compounds including most drugs.[22, 25 – 29, 147, 339, 345, 346, 359, 361] With the participation of the CYP1A2 enzyme, about 80% of caffeine is metabolized by 3-demethylation to paraxanthine. The CYP1A2 and CYP2E1 enzymes are involved in 1- and 7-demethylation of caffeine to give theobromine (12%) and theophylline (7%), respectively. In addition, caffeine was proposed as a safe biomarker for evaluating the condition of the liver at various diseases, because the principal caffeine-metabolizing enzyme CYP1A2 was detected in the liver.[25, 139, 339, 358, 365, 368, 369] The standard procedures for estimating the cytochrome P450 enzyme activity includes determination of caffeine and three key metabolites in urine, blood plasma or saliva some period after caffeine intake.

In most publications, methylxanthines have been determined in biological fluids by reversed-phase HPLC with UV or MS detection. Most often, analysis for caffeine and theophylline is performed, while theobromine,[18, 28, 29, 146, 184, 189, 191, 235] diprophylline,[355] pentoxyphylline[156, 334, 355] and paraxanthine[28, 146, 191] are quantified more rarely. Using HPLC, it is possible to determine methylxanthines in biological fluids at the level of hundredths of microgram per millilitre. For example, it was reported[146] that the detection limits of theobromine, paraxanthine, theophylline and caffeine in urine (the compounds are listed in the order of elution) are 0.03, 0.02, 0.04 and 0.08 μg ml^{-1}, respectively. This method was used to estimate the contents (given in parentheses, μg ml^{-1}) of caffeine (0.06 – 0.77), theophylline (0.1 – 0.66), theobromine (0.08 – 0.50) and paraxanthine (0.15 – 1.68) in breast milk.[191] Other versions of HPLC and also various versions of electrophoretic methods (see Table 6) are not so widely used.

Methylxanthines are determined by gas chromatography both as individual compounds[157, 159, 183] and as volatile derivatives after derivatization with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane.[365]

A mass-spectrometric [159, 183, 365] or thermionic [157, 234] detector is used. The detection limits of caffeine were 1 ng ml^{-1} for determination in blood plasma and saliva [157] and 10 ng ml^{-1} for determination in sweat;[159] the theophylline detection limit for blood serum was 0.07 μg ml^{-1} (Ref. 183). The detection limit of caffeine in newborn nails turned out to be 0.025 ng ml^{-1} (Ref. 365). The detection limits of caffeine, theophylline, theobromine and paraxanthine after their solid-phase microextraction from urine and blood were 0.06–0.9 μg ml^{-1} (Ref. 234).

The spectrophotometric determination of methylxanthines in biological specimens is often combined with mathematical treatment of the results.[17, 96, 153] Samples are prepared by various procedures. In a publication,[153] theophylline was determined in blood plasma after extraction into a dichloromethane–isopropyl alcohol mixture. In another work,[17] determination of theophylline in blood serum was preceded by precipitation of proteins with trichloroacetic acid. In another work,[95] the sample was only diluted for determination of caffeine in blood serum. The detection limit for caffeine was 0.04 μg ml^{-1}. For theophylline determination in blood serum, a kinetic spectrophotometric method was proposed based on the detection of the signal of the product of theophylline oxidation with cerium(IV) sulfate.[348] The determination of theophylline is not interfered with by caffeine or other methylxanthines, as the oxidation affects the unsubstituted hydrogen atom of the imidazole ring of theophylline. The detection limit is 4.5 μ mol litre^{-1}. Indirect determination of theophylline in blood serum is based on fluorimetric monitoring of 4-methylumbelliferone the hydrolysis of which is inhibited by theophylline. The detection limit of theophylline is 0.1 mmol litre^{-1} (Ref. 349).

A number of sensors have been developed for selective determination of methylxanthines in biological specimens.[352–357, 364] The caffeine sensor based on a caffeine molecular imprinted polymer is suitable for caffeine determination in blood serum and urine with a detection limit of 5×10^{-9} mol litre^{-1} (Ref. 352). Biosensors for determination of caffeine [364] and theophylline [356] have been designed. A high potential is expected of immunochemical methods for theophylline determination based on the use of specific anti-theophylline antibodies. Using immunosensors, determination of theophylline in blood serum was performed with detection limits of 0.3 to 5 ng ml^{-1} (Refs 353–355).

2. Determination in environmental specimens

The interest in determination of pharmaceutical agents in environmental specimens has enhanced in the last decade. These compounds and their metabolites, together with surfactants, personal hygiene products and other substances related to activities of daily living, enter the environment as complex mixtures, most often as waste water components.[197] A HPLC analysis followed by mass-spectrometry detection of 164 underground water samples of 23 European countries was carried out.[370] The sample preparation included solid-phase extraction using the cartridge Oasis HLB (200 mg). Among the 59 identified compounds, the compounds listed in Table 8 were found in the samples most often.

Table 8. Detection frequency (n) and maximum concentration (c_{max}) of the most abundant compounds in underground waters.[370]

Compound	n (%)	c_{max} /ng litre^{-1}
Diethyltoluamide	84	454
Caffeine	83	189
Perfluorooctanoic acid	66	39
Atrazine	56	253
Deethylatrazine	55	487
1*H*-Benzotriazole	53	1032
Methylbenzotriazole	52	516
Perfluorooctanesulfonate	48	135
Simazine	43	127
Carbamazepine	42	390
Nonylphenoxyacetic acid	42	11 [a]
Bisphenol A	40	2.3 [a]
Perfluorohexanesulfonate	35	19
Bentazone	32	11 [a]
Propazine	32	25
Perfluoroheptanoic acid	30	21
2,4-Dinitrophenol	29	122
Diuron	29	279
Sulfamethoxazole	24	38

[a] In μg litre^{-1}.

Caffeine was detected in surface and waste waters in many countries including Germany,[201, 371] France,[196] Sweden,[38] Canada,[40, 372] Spain,[195, 197, 199, 373, 374] Greece,[34] Romania,[202] Ireland,[203] USA,[198, 375, 376] Brazil,[204, 222] Taiwan [377, 378] and Korea.[379] Furthermore, it was detected in underground water,[34, 370, 380, 381] potable water,[204, 382, 383] rain water and mountain spring water [384] and in seawater.[36, 40] Systematic research into the caffeine distribution showed that its content in crude waste water varies from 7 to 73 μg litre^{-1} and in treated waste water it was 0.03 to 9.5 μg litre^{-1} (Refs 38, 381, 385). Therefore, it was proposed to use caffeine as an analytical marker for monitoring domestic waste water discharge to the environment.[38, 202, 371, 372, 381] Apart from caffeine, environmental specimens are analyzed for pentoxyphylline,[143, 194, 202] theobromine and theophylline.[222]

In most of publications, caffeine and other methylxanthines were determined simultaneously with other drug substances. The key determination methods include gas chromatography [36, 40, 195, 197, 201, 202, 204, 371, 385, 386] and HPLC [195, 198, 203, 370, 375, 380, 382–384, 387, 388] followed by mass spectrometric detection and, more rarely, HPLC with UV detection.[34, 199, 222, 373, 374, 381] Determination was preceded by sample preparation, which was carried out most often by solid-phase extraction with various cartridges: StrataX,[204, 205] Bakerbond SDB-1,[36] LiChrolut EN [34] Oasis HLB.[40, 195, 199, 201, 202, 370, 374, 380, 383, 384, 388] In some cases, cartridges filled with C_{18},[195, 196] a molecularly imprinted polymer [222] or carbon [381] and liquid – liquid extraction were used.[385] These procedures ensure the following detection limits for caffeine determination: 0.036–5 ng litre^{-1} (gas chromatography with mass spectrometric detection), 0.014–0.138 μg litre^{-1} (HPLC with mass spectrometric detection) and 0.03–0.05 μg litre^{-1} (HPLC with UV detection). An immunosensor was designed [389] suitable for caffeine determination in river water without sample preparation with a detection limit of 1 ng litre^{-1} .

Pentoxyphylline was determined in waste water [194, 202] by gas chromatography followed by mass spectrometry detection after sorption preconcentration of the compound on C_{18} and Oasis HLB cartridges; the detection limits were

1.7 and 10 ng ml^{-1}, respectively. Whereas crude waters contained up to 299 ng $litre^{-1}$ of pentoxyphylline, after water treatment this compound was not detected.[202] Theophylline and theobromine were identified in waters.[222] Their detection limits for HPLC with UV detection and solid-phase extraction on the molecularly imprinted polymer were 44 and 85 μg $litre^{-1}$, respectively.

Apart from various types of water, caffeine together with other drug substances and personal-care product components are determines in soils[140] and silt.[143] The sample preparation comprised successive liquid extraction from solid matrices with dichloromethane and acetone[140] or with methanol and acetone assisted by sonication.[143] The detection limit of caffeine in soil was 0.11 ng g^{-1} (gas chromatography/mass spectrometry),[140] and those for caffeine and pentoxyphylline in silt are 50 and 20 ng g^{-1}, respectively (HPLC with UV detection).[143]

VI. Conclusion

The considerable interest in the development of methods for determination of methylxanthines in various objects is due to the importance of these compounds for pharmacology, clinical and sports medicine, toxicology and environmental control. Currently, determination of relatively large concentrations of methylxanthines present in food products and medicaments causes almost no problems. During the last 15 years, a large number of methods for analysis of such specimens have been developed.

Determination of these compounds in biological fluids is faced with more problems due not only to low concentrations of methylxanthines but also to the complex composition of the samples. Often it is required to determine methylxanthines together with other compounds present as components of these materials. The gas chromatography/mass spectrometry method has the highest potential for multicomponent analysis, as it makes it possible to identify compounds based both on the retention time and the general mass spectrum and molecular mass. Apparently, studies concerned with determination of methylxanthines in biological specimens would be further developed due to the considerable interest aroused lately by metabolomics, *i.e.*, construction of the global concentration profile for all metabolites in the human body.[390, 391] Metabolomics has already proved itself to be efficient for the detection of inborn and hereditary metabolic disturbances caused by endogenic and exogenic factors, for organ transplantation (before and after the surgery), for investigation of the toxicity of medicaments (toxicogenomics) and body response to medicaments (pharmacogenomics), for determination of personal peculiarities of the body response to food items (nutrinomics). The principal pathologies that are within the scope of metabolomics include metabolic syndrome, diabetes, cardiovascular diseases, liver pathologies. The knowledge about the concentrations of metabolites of caffeine and other methylxanthines in the human body is a significant aspect for the construction of such profiles.

The considerable interest in the determination of caffeine and other methylxanthines in environmental specimens is due to the need to monitor the contents of drug substances in them.[392, 393] In this connection, development of new rapid techniques for sample preparation will apparently continue, as right choice of these techniques is often responsible for both reliability of identification of methylxanthines and correctness of the quantitative results. It is important to look for new sorbents that would provide, on the one hand, quantitative preconcentration of methylxanthines together with other compounds and, on the other hand, selective recovery of these compounds. As shown by recent studies, a promising type of such sorbents are molecularly imprinted materials, which have already found use in the analytical chemistry of methylxanthines (in solid-phase extraction and HPLC methods) and in the manufacture of sensitive elements of sensors. The role of immunochemical methods for methylxanthine analysis, which are highly sensitive, specific, fast and suitable for simultaneous analysis of a large number of samples, is ever increasing.

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