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Simultaneous determination of caffeine, theobromine, theophylline, paraxanthine and nicotine in human milk by liquid chromatography with diode array UV detection

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Abstract

A liquid chromatography-diode array UV detection (LC–UVDAD) method for the simultaneous determination of the alkaloids Nicotine, Caffeine, Theobromine, Paraxanthine and Theophylline is described. The chromatographic separation was achieved on a LC-18-DB column using 20:80 methanol:buffer (5 mM sodium octane sulphonate, 10 mM citric acid adjusted to pH 5.8 with triethylamine) as mobile phase. The method has been applied to human milk samples. The overall procedure had % recoveries ranging from 60.2 ± 0.6 (theobromine) to 98.6 ± 0.4 (caffeine). The within-day ($n = 5$) and between-days ($n = 5$ over 5 days) coefficients of variation in milk ranged from 3.7% (caffeine) to 4.7% (theobromine) and from 5.1% (caffeine) to 6.7% (theobromine). Estimated LOD and LOQ in milk ranged from 8 (caffeine) to 13 (nicotine) ng/ml and from 24 (caffeine) to 34 (nicotine) ng/ml, respectively. Samples naturally contaminated with the target analytes were found.
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Keywords: LC; Nicotine; Caffeine; Theobromine; Paraxanthine; Theophylline; Human milk

1. Introduction

It is well recognized that although nutritionally breast milk is the optimal food for babies, there are a number of caveats to this, based on the consequence of modern lifestyle. Infants consuming breast milk may be exposed to a variety of chemicals which may have untoward effects on his immediate health and temperament and future development (Campoy et al., 2001). These substances can be grouped in the following areas: medication taken by the mother; exposure to possibly addictive drugs taken by the mother; exposure to pollutants mainly from the maternal diet. The substances ingested by the lactating mother are passed through the epithelial cells of the mammary glands to the milk. The rate of diffusion across the mammary glands may be modulate by the physiochemical properties of the molecules and their concentration in the milk depends on the dose ingested, the duration of the assumption, the amount of milk excreted daily, the mother's health and her genotype.

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthyne) are the most important naturally occurring methylxanthynes. They are natural alkaloids and show various physiological effects (Eteng, Eyong, Akapanyung, Agiang, & Aremu, 1997) on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems. Caffeine is a constituent of coffee and other beverage. Theobromine and theophylline are presents in cocoa, tea and chocolate products. Theophylline is also a widely used bronchodilating agent with a narrow serum therapeutic range. Paraxanthine (1,7-dimethylxanthine) is not found in foods but is the main metabolite of caffeine. Nicotine,

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responsible for tobacco addiction, is the most specific component of cigarette smoke and has been suspected (Benowitz, 1997) to contribute to human disease like cardiovascular and reproductive disorders. There is also substantial evidence (Golding, 1997) to show that smoking mothers who breast feed are exposing their children to nicotine with a concentration up to 114 mg/l. That this is adsorbed by the infant is shown by a number of studies, with consequent short term symptoms of restlessness, insomnia, nausea, vomiting, diarrhoea, rapid pulse, etc. and possible long term symptoms of physical and mental handicap.

Even when the presence of the above contaminants in human milk is slight, it may affect the health of breast fed infants since they are more sensitive than adults. Thus, a selective and sensitive method for their simultaneous determination in human milk is highly advisable, since analytical screens that can detect multiple analytes in one assay are vital for rapid laboratory response.

Chromatographic methods for the quantification of some of these analytes have been developed in urine (Delbeke & De Backer, 1996) for theophylline, chocolate (Caudle, Gu, & Bell, 2001) for theobromine and caffeine, blood and urine (Kumazawa et al., 1999) for caffeine, theobromine, paraxanthine, theophylline and meconium (Baranowski, Pochopien, & Baranowska, 1998) for caffeine and nicotine. Applications to human milk are essentially lacking with the exception of two paper (Ilett et al., 2003; Page-Sharp, Hale, Hackett, Kristensen, & Ilett, 2003) dealing with the LC–UV determination of nicotine and cotinine.

In the present work, a LC–UV/DAD method for the simultaneous determination of the alkaloids Nicotine, Caffeine, Theobromine, Paraxanthine and Theophylline (see Fig. 1) was developed for the first time. The eluent composition was properly optimized in order to achieve the highest possible chromatographic resolution. The method possess wide linearity and sufficiently low detection limits. The applicability of the procedure to the analysis of real samples was demonstrated by the analysis of human milk samples.

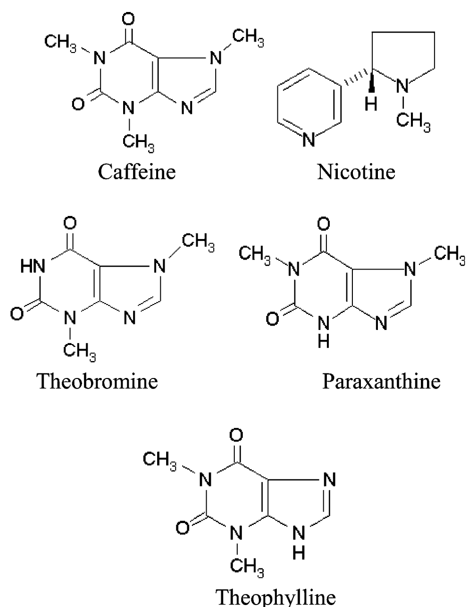

Fig. 1. Chemical structure of the target alkaloids.

2. Experimental

2.1. Chemicals

Nicotine, caffeine, theobromine, paraxanthine and theophylline were obtained from Sigma (St. Louis, MO, USA). Stock solution were prepared in tridistilled water and stored at 4 °C in the dark. Dilute solutions were prepared just before use.

Organic solvents (Carlo Erba, Milan, Italy), were HPLC grade. Mobile phase was filtered through a 0.45-μm membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The HPLC system consisted of a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), equipped with a Rheodyne injector with a 100 μl loop and a 5-μm Supelcosil LC-18-DB column (250×4.6 mm i.d.) (Supelco). A 5-μm Supelguard LC-18-DB precolumn (20×4.6 mm i.d.) (Supelco) was used to protect the analytical column. The detector was a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The composition of the optimized buffer was 5 mM sodium octane sulphonate, 10 mM citric acid adjusted to pH 5.8 with triethylamine. The optimized mobile phase conditions were 20:80 methanol:buffer with a flow rate of 1 ml min^{-1} at ambient temperature. Mobile phase was degassed on-line by an SCM1000 Vacuum Membrane Degasser (Thermo Separation Products). Spectra were acquired in the 230–380 nm range using 1 Hz frequency and 5 nm bandwidth. Chromatograms were monitored at 260 nm (10 Hz frequency, 5 nm bandwidth).

2.4. Sample collection and pre-treatment

Milk samples were collected by hand expression or manual breast pump from 10 non-smoking nursing mothers; six of them were habitual coffee and chocolate consumers. These samples were stored at 4 °C for not over 2 days before analysis.

A 3.5-ml aliquot of milk was added to 4.0 ml of TCA 20% solution and centrifuged at 5000*g* for 15 min. The supernatant was transferred to a clean glass tube and adjusted to pH 5 with ammonia buffer. The resulting mixture was loaded onto a previously conditioned (elution of 2 ml of MeOH and 2 ml of H_2O) ultra-clean solid phase extraction tube (Alltech, Deerfield, IL, USA) containing 200 mg of a 50-μm particle size C_{18} packing. The SPE tube was connected to a syringe filter containing a 3-mm diameter, 0.45 μm pore size nylon membrane (Supelco). The SPE tube was then washed with 2 ml of 10 mM citric acid, adjusted to pH 5.8 with triethylamine, and eluted three folds with 200 μl of 50% (v/v) methanol in 10 mM citric acid, adjusted to pH 5.8 with triethylamine. First 200 μl were discarded and 100 μl of the final eluate were injected.

Recoveries were calculated as peak area ratio of analyte (standard)/analyte (spiked milk samples). Milk samples were spiked with the analytes at 0.1, 0.5 and 1 μg/ml concentration levels, equilibrated at room temperature for at least 1 h and then analyzed as described above.

Quantitation was performed with the standard addition method. Calibration curves in milk were constructed spiking analyte free human milk samples with Nicotine, Caffeine, Theobromine, Paraxanthine and Theophylline in order to cover the range from 0.04 to 2 μg/ml; five replicates for each concentration were performed.

The within-day ($n = 5$) and between-days ($n = 5$ over 5 days) coefficient of variation in milk were calculated on analyte free milk samples spiked with variable amounts of the target analytes in order to obtain the following concentration levels: 0.1, 0.5 and 1 μg/ml.

3. Results and discussion

The first step of the present work was to investigate factors influencing retention and chromatographic efficiency of the target compounds, i.e. pH, organic modifier and ion pairing agent content, in order to optimize their separation.

Fig. 2 reports the LC–UV chromatograms obtained by direct injection of a standard solution of nicotine, caffeine, theobromine, paraxanthine and theophylline at a concentration level of 0.5 μg/ml using the optimised mobile phase composition, i.e. 20:80 methanol:buffer (5 mM sodium octane sulphonate, 10 mM citric acid adjusted to pH 5.8 with triethylamine) and shows its capability to resolve the target compounds. Under these conditions theoretical plates number ranging from ca. 9000 m^{-1} (nicotine) to ca. 19,700 m^{-1} (theophylline) and good peak symmetry factors (Foley & Dorsey, 1983) were achieved.

Once the ideal chromatographic conditions were reached, the method was applied to the determination of nicotine, caffeine, theobromine, paraxanthine and theophylline in human milk samples.

Fig. 3 shows, for instance, the LC–UV chromatograms relevant to (A) a analyte free milk sample and

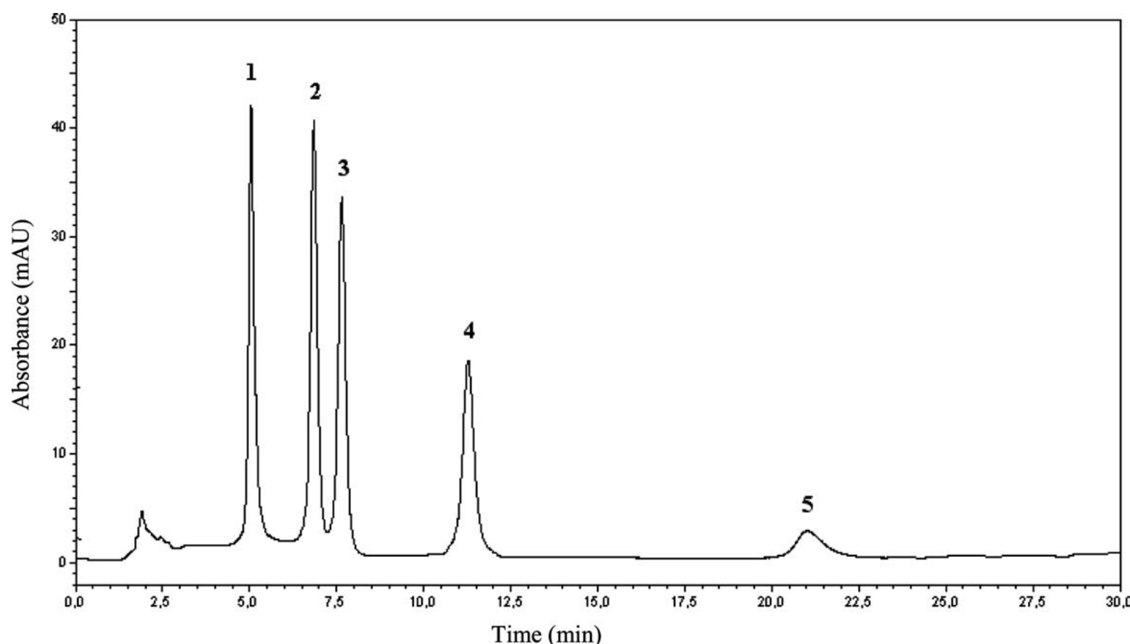

Fig. 2. LC–UV chromatogram obtained by direct injection of a standard solution of the target analytes at a concentration level of 0.5 μg/ml. Peak legend: **1**, theobromine; **2**, paraxanthine; **3**, theophylline; **4**, caffeine; **5**, nicotine.

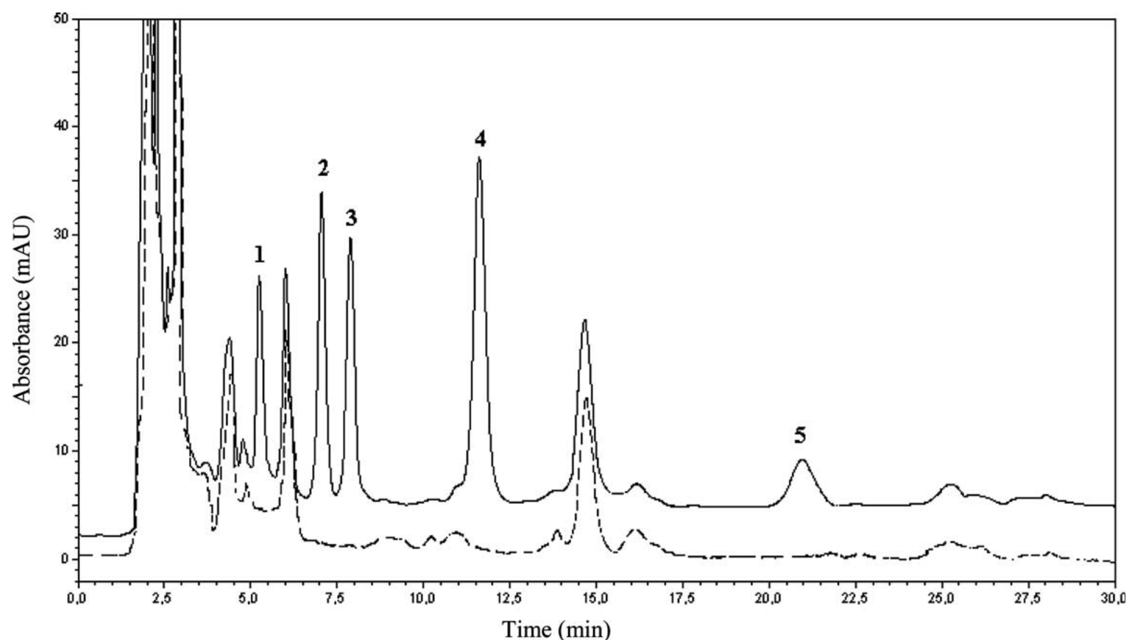

Fig. 3. LC–UV chromatograms relevant to: (A) a analyte free milk sample and (B) the same sample spiked with known amounts (1 μg/ml) of the target analytes, respectively, and treated as described in the experimental section. *Peak legend*: **1**, theobromine; **2**, paraxanthine; **3**, theophylline; **4**, caffeine; **5**, nicotine.

(B) the same sample spiked with known amounts (1 μg/ml) of the target analytes, respectively, and treated as described in Section 2. As apparent, the optimized mobile phase permitted to resolve the target compounds with no significance interference from milk endogenous components.

Calibration curves resulted linear in the range 0.04–2 μg/ml with correlation coefficients better than 0.998 and intercept not significantly different from zero at 95% confidence level.

The within-day ($n = 5$) coefficients of variation, estimated by an ANOVA test, were: 3.7% (caffeine), 4.7% (theobromine), 3.8% (theophylline), 4.5% (paraxanthine) and 4.1% (nicotine). The between-days ($n = 5$ over 5 days) coefficients of variation, estimated by an ANOVA test, were: 5.1% (caffeine), 6.7% (theobromine), 5.5% (theophylline), 6.0% (paraxanthine) and 5.8% (nicotine). All coefficients of variation remained practically unchanged passing from 0.1 to 1 μg/ml level.

The obtained % recoveries ± standard deviation ($n = 5$) were 98.6 ± 0.4 (caffeine), 60.2 ± 0.6 (theobromine), 60.4 ± 0.5 (theophylline), 68.3 ± 0.6 (paraxanthine) and 74.4 ± 0.6 (nicotine), respectively, and remained practically unchanged passing from 0.1 to 1 μg/ml level.

LOD and LOQ were calculated according to Eurachem (Vial & Jardy, 1999), as the analyte concentration giving an RSD = 20 and 12%, respectively. The estimated LOD were in the ranges 8 (caffeine) and 13 (nicotine) ng/ml, respectively, while LOQ were in the range 24 (caffeine) and 34 (nicotine) ng/ml, respectively.

Table 1
Alkaloids concentrations estimated in the considered milk samples

compound	Concentration (μg/ml)					
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 5	Milk 6
Nicotine	–	–	–	–	–	0.06
Caffeine	0.66	0.06	0.20	0.18	0.07	0.77
Theobromine	0.20	0.50	0.10	0.08	0.46	0.19
Paraxanthine	0.40	0.15	0.20	0.20	1.68	0.34
Theophylline	0.14	0.60	0.10	0.10	0.66	0.12

Six of the 10 analyzed samples were found to be contaminated with variable amounts of the investigated analytes. Peak identity was confirmed on the basis of retention times and by UV spectra overlay. Table 1 reports the relevant results. As apparent, one sample was found contaminated with all the target analytes. Fig. 4 shows the relevant LC–UV chromatogram. The inset in Fig. 4 reports a portion of the chromatogram of the same milk sample spiked with nicotine standard at a known concentration level (0.1 μg/ml).

It is worth noting that since all the donors were nonsmoking subjects and habitual coffee and chocolate consumers, the presence of caffeine, theobromine, paraxanthine and theophylline was not surprising; on the contrary, the presence of nicotine was unexpected and could be due to the exposition of the donor to passive smoke.

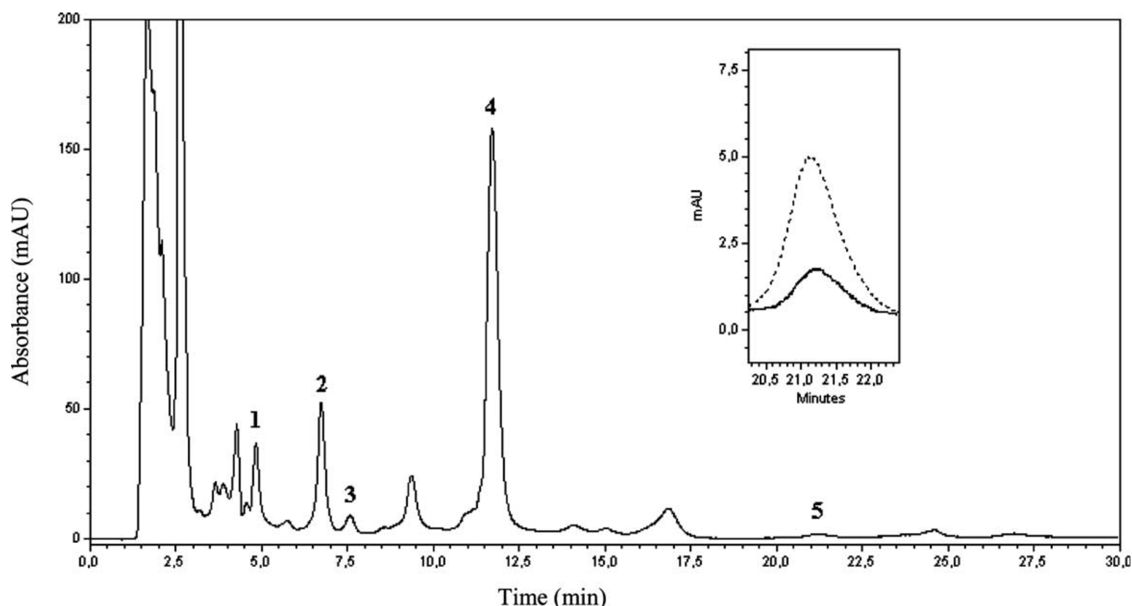

Fig. 4. LC–UV chromatogram relevant to the analysis of a human milk sample (milk 6) naturally contaminated with the target alkaloids (see Table 1 for the estimated concentration). The inset shows a portion of the chromatogram of the same milk sample spiked with nicotine standard at a known concentration level (0.1 μg/ml). *Peak legend*: **1**, theobromine; **2**, paraxanthine; **3**, theophylline; **4**, caffeine; **5**, nicotine.

4. Conclusions

A LC–UV method for the simultaneous determination of nicotine, caffeine, theobromine, paraxanthine and theophylline has been developed for the first time. The LC separation of the target compounds could be achieved on a LC-18-DB column using 20:80 methanol:buffer (5 mM sodium octane sulphonate, 10 mM citric acid adjusted to pH 5.8 with triethylamine) as mobile phase. The potential of the described procedure was demonstrated by the application to human milk samples naturally contaminated with the target analytes.

The method could be potentially applied to the determination of the target alkaloids in other matrices, such as biological fluids. Work is in progress in this direction.

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