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Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography–electrospray tandem mass spectrometry

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ABSTRACT

A liquid-chromatography–tandem-mass-spectrometry method using pneumatically assisted electrospray ionisation (LC–ESI-MS/MS) was developed for the simultaneous determination of cathinone, methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylone, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine in human live and post-mortem whole blood. The blood proteins were precipitated by the addition of methanol, and the extract was purified by ultrafiltration. The separation of diastereomeric ephedrines was achieved on an ethyl-linked phenyl column. Matrix-matched calibrants combined with the isotope dilution of selected substances were used for quantitative analysis. The relative intra-laboratory reproducibility standard deviations were generally better than 7% at concentrations of 20 μg/L, and the mean true recoveries were 87–106% in the concentration range of 10–250 μg/L. The detection limits were in the range of 0.5–3 μg/L. The cathinones were unstable in whole blood and sample extracts under neutral conditions, but the stability could be improved by the acidification of the sample matrix.

1. Introduction

Leaves from the scrub khat (*Catha edulis*) have been chewed for centuries by people from Africa and the Arabian Peninsula because of their stimulating effect. Due to migration, the use of khat has spread and gained an increasing global prominence. The stimulating effect arises from the alkaloid content of the phenylalkylamine type. The main phenylalkylamines found in khat leaves are S-(−)-cathinone (Table 1) and the two diastereomers 1S,2S-(+)-norpseudoephedrine (cathine) and 1R,2S-(−)-norephedrine (Table 2). Cathinone is considered to be the main psychoactive constituent of khat, and it produces an effect similar to that of amphetamines. It is found primarily in young leaves. During leaf maturation, the cathinone concentration declines, which may be caused by the biochemical reduction of cathinone to cathine and 1R,2S-(−)-norephedrine [1]. In humans, cathinone is mainly metabolised into 1R,2S-(−)-norephedrine [2].

The presence of cathine and norephedrine in body fluids is not an unambiguous indicator of khat chewing. The metabolism of pseudoephedrine, which commonly is used in over-the-counter cold and allergy formulations, also produces cathine. Similarly, ephedrine, which is used as a decongestant, is metabolised to norephedrine. Furthermore, cathine and norephedrine may still be available in some countries as ingredients in anorectic products, and norephedrine may still be found in cold medications. The presence of ephedrine alkaloids in blood may also originate from the intake of nutritional supplements containing plant material or extracts of *Ephedra* species. Both cathine and 1R,2S-(−)-norephedrine are found in *Ephedra* species [3,4]. To detect khat chewing with high probability, cathinone should be present in body fluids and preferably in the absence of N-alkylated derivatives such as amfepramone, ethcathinone and methcathinone because they could theoretically be metabolised to cathinone [5].

In addition to the native cathinone available from the khat plant, a broad range of cathinone derivatives including methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylone and ethylone has been synthesised (Table 1). They are mainly used for their psychoactive effects. However, amfepramone, which is metabolised to ethcathinone, is a stimulant drug that is legally available in many countries as an appetite suppressant.

In forensic toxicology, body fluids are monitored for illicit substances and therapeutic drugs that may have been abused. This process is typically performed in connection with road-site testing of vehicle drivers and in cases of violent crime, poisonings and suspicious death. The therapeutic blood concentration ranges of amfepramone, ephedrine and pseudoephedrine span the intervals of 0.007–0.2, 0.02–2 and 0.5–0.8 mg/L, respectively [6].

Testing for ephedrines and cathinones is typically performed by GC–MS in urine and other matrices. However, the use of LC–MS or LC–MS/MS allows the derivatisation step to be excluded. Methods based on LC–MS/MS have been published for the determination of

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Table 1
Cathinones included in the validated method.

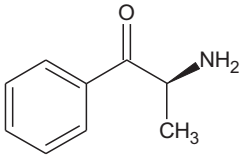
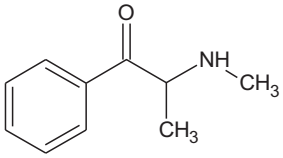
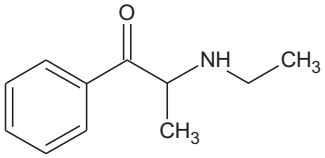
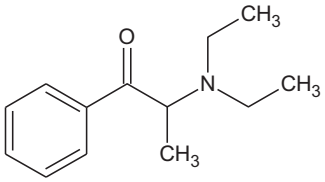
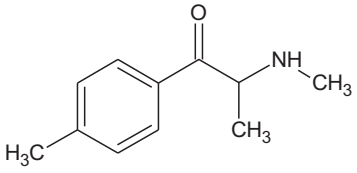
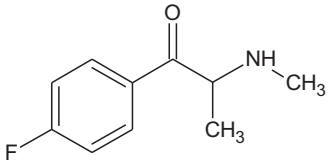
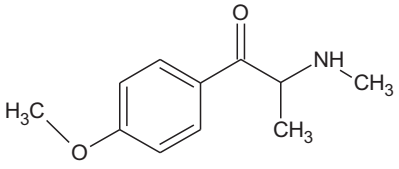
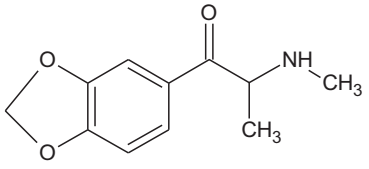
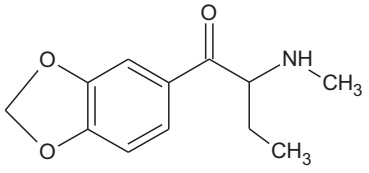
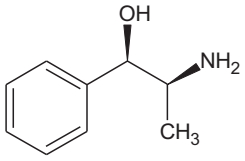
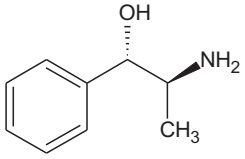
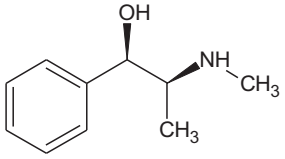
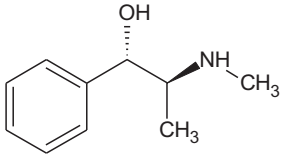
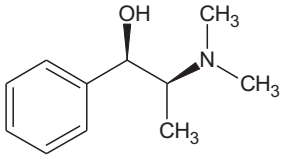
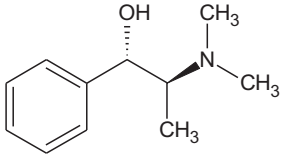
Substance	Molecular structure
S-(−)-cathinone	
Methcathinone	
Ethcathinone	
Amfepramone (diethylcathinone, diethylpropion)	
Mephedrone (4-methylmethcathinone)	
Flephedrone (4-fluoromethcathinone)	
Methedrone (βk-PMMA, 4-methoxymethcathinone)	
Methylone (βk-MDMA, 3,4-methylenedioxymethcathinone)	
Butylone (βk-MBDB)	

Table 2
Ephedrines included in the validated method.

Substance	Molecular structure
1R,2S-(−)-norephedrine	
Cathine (1S,2S-(+)-norpseudoephedrine)	
1R,2S-(−)-ephedrine	
1S,2S-(+)-pseudoephedrine	
1R,2S-(−)-methylephedrine	
1S,2S-(+)-methylpseudoephedrine	

ephedrines in urine [7,8] and plasma [7,9] and for the determination of cathinone in urine [10] and plasma/serum [9,11].

The present LC–MS/MS method was developed and validated to obtain a simple technique for the simultaneous identification and quantification of the major alkaloids from khat, their precursors from the ephedrine group and selected cathinone derivatives in live and post-mortem whole blood.

2. Experimental

2.1. Chemicals and reagents

The ephedrines 1R,2S-(−)-ephedrine HCl, 1S,2S-(+)-pseudoephedrine, 1R,2S-(−)-norephedrine, 1R,2S-(−)-methylephedrine and 1S,2S-(+)-methylpseudoephedrine were purchased from Sigma–Aldrich (Schnelldorf, Germany). Methcathinone HCl and cathine HCl were obtained from Lipomed AG (Arlesheim, Switzerland). Mephedrone HCl, flephedrone HCl, methedrone HCl, methcathinone-D3 HCl, cathine-D3 HCl and mephedrone-D3 HCl were obtained from Toronto Research Chemicals Inc (North York, Canada). Amfepramone HCl was obtained from Temmler Pharma GmbH & Co. KG (Marburg, Germany). Cathinone HCl, ethcathinone HCl, methylone HCl, butylone HCl and 3-fluoromethcathinone HCl were obtained from the Australian Government National Measurement Institute (Sydney, Australia). norephedrine-D3 HCl, pseudoephedrine-D3 HCl and 1S,2R(+)-ephedrine-D3 HCl were obtained from Cerilliant (Round Rock, TX). Blank whole-blood samples for calibration were obtained from the Blood Bank, Aarhus University Hospital (Skejby, Denmark). The blank samples of live and post-mortem whole blood used for the method validation were obtained from the Institute of Forensic Medicine, University of Aarhus. Live blood was collected and preserved in Venosafe tubes containing a mixture of sodium fluoride (NaF) and potassium oxalate (Terumo Europe, Leuven, Belgium). Post-mortem blood samples were preserved with 200 mg of NaF per 30 mL of blood. Venosafe tubes (Terumo Europe) containing mixtures of NaF and citrate buffer ingredients were included in a stability study on cathinones in live whole blood. Formic acid and methanol (MeOH) were purchased from Merck. Water was purified using a Direct-Q 3 apparatus (Millipore, Bedford, MA).

Separate stock solutions containing 1 mg/mL of the active substances were prepared in MeOH. The combined standard solutions for fortification of samples and preparation of the calibrants were prepared by diluting the stock solutions with MeOH. An internal standard solution (IS) containing 500 μg/L of the deuterated analogues of methcathinone and mephedrone and 1500 μg/L of the deuterated analogues of ephedrine, norephedrine, pseudoephedrine and cathine was also prepared in MeOH.

The mobile phases 1A and 1B consisted of water and MeOH, respectively, which were both acidified with 0.1% formic acid.

2.2. Equipment

The liquid-chromatographic system was a Waters 2695 Separations Module consisting of a binary pump, a solvent degasser, an autosampler with a sample compartment thermostatted at 10 ± 2 °C and a column oven thermostatted at 30 ± 2 °C (Waters, Milford, MA). The mass spectrometer was a Micromass Quattro Micro API triple-quadrupole instrument with an ESCi ion source (Waters). The separation was performed on a Prodigy Phenyl-3 (5 μm, 2.0 mm I.D. × 150 mm) column (Phenomenex, Torrance, CA) connected to a Phenyl SecurityGuard pre-column cartridge (2.0 mm I.D. × 4 mm) (Phenomenex). Amicon Ultra units with a 0.5-mL reservoir and a 30-kDa membrane of regenerated cellulose (Millipore, Bedford, MA) were used for the ultrafiltration (UF) of the sample extracts. Disposable 2-mL Safe-Lock tubes in polypropylene (Eppendorf, Hamburg, Germany) were used for the extraction. Crimp-top autosampler vials with an integrated 300-μL insert were obtained from Chromacol (Welwyn Garden City, UK). Other equipment used included pipettes (Biohit, Helsinki, Finland) and a Heraeus Biofuge Pico (Thermo Scientific, Langenselbold, Germany).

2.3. Extraction

A 300-μL volume of blood was mixed with 100 μL of IS solution in a disposable 2-mL centrifuge tube. A 600-μL volume of MeOH was added, and the tube was immediately closed and vortex-mixed for few seconds. After a standing time of 10 min, the mixture was centrifuged at 10,000 × *g* for 5 min. A 300-μL volume of clear supernatant was transferred to a UF filter cup, and 10 μL of formic acid was added. The unit was centrifuged at 10,000 × *g* for 10 min, and 100 μL of the filtrate was mixed with 100 μL of water in an autosampler vial.

2.4. Calibration

Calibrants based on blank donor blood from single persons were used for the construction of 5-point calibration curves. The samples were treated according to the procedure except that 100 μL of MeOH was replaced by 100 μL of the mixed standards of the drug

Table 3
Internal standards (IS) used for quantification.

Drug	IS	Transition Q1/Q3 (*m*/*z*)
Norephedrine	Norephedrine – D3	155/137
Cathine	Cathine – D3	155/137
Ephedrine	Ephedrine – D3	169/151
Pseudoephedrine	Pseudoephedrine – D3	169/151
Cathinone	Ephedrine – D3	169/151
Flephedrone	Methcathinone – D3	167/149
Methcathinone	Methcathinone – D3	167/149
Methylephedrine	Pseudoephedrine – D3	169/151
Methylpseudoephedrine	Pseudoephedrine – D3	169/151
Ethcathinone	Mephedrone – D3	181/163
Methylone	Mephedrone – D3	181/163
Methedrone	Mephedrone – D3	181/163
Mephedrone	Mephedrone – D3	181/163
Butylone	Mephedrone – D3	181/163
Amfepramone	Mephedrone – D3	181/163

substances; sample concentrations were obtained at 10, 100, 200, 300 and 400 μg/L of cathinones and 30, 300, 600, 900 and 1200 μg/L of ephedrines in the original blood sample. The calibration curves were created by weighted (1/×) linear regression analysis on the IS-normalised peak areas (analyte area/IS area) and were forced through the origin. The internal standards used for the different substances are listed in Table 3.

2.5. LC–MS/MS conditions

The sample extracts were kept at 10 ± 2 °C until analysis. A 10-μL volume was injected onto a Prodigy Phenyl-3 column running 95% mobile-phase A and 5% B. After 1 min, a linear gradient was used to change the mobile phase to 30% A and 70% B over 19 min. Then the gradient was changed to 90% B over 0.5 min. After a total time of 22 min, the gradient was returned to 5% B over 0.5 min, and the column was equilibrated for 5 min before the next injection. The eluent was diverted to waste during the intervals 0–4 min and 19–28 min after injection using a post-column switch. The column flow rate was 200 μL/min, and the column temperature was maintained at 30 ± 1 °C. The source and desolvation temperatures were set at 140 °C and 350 °C, respectively, and the cone and desolvation gas flows were set at 50 L/h and 800 L/h, respectively. The mass spectrometer was operated in positive ion mode with a probe voltage of 4000 V and an extractor potential of 3 V. The dwell time was 200 ms for all ion transitions. Selected reaction monitoring (SRM) was applied under the conditions shown in Table 4. Argon was used for collision-induced dissociation (CID). The data acquisition and processing were performed using MassLynx 4.1 (Waters). Unscrambler 9.2 (Camo, Trondheim, Norway) was used for chemometric data exploration.

2.6. Limit of detection

The limits of detection (LODs) were determined using a random selection of 40 different blank control samples of live and post-mortem whole blood (20 of each type). The samples were fortified prior to extraction with the individual substances to obtain concentrations that were approximately three times the signal/noise ratio. The LODs were calculated as three times the standard deviation (SD) of the measured results.

2.7. Precision, trueness and recovery

The repeatability standard deviation (SD_r) (i.e., the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation ($SD_{R,intra\text{-}lab}$) (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different experimental conditions) were determined on blank control samples of live and post-mortem whole blood fortified to levels of 4, 20 and 200 μg/L of cathinones and 12, 60 and 600 μg/L of ephedrines. Duplicate analyses were performed on eight different days. The repeatability and intra-laboratory reproducibility parameters were calculated in accordance with ISO standard 5725-2 [12].

The ion-suppression effects were investigated on 20 blank samples of both live and post-mortem blood fortified after extraction and UF to a level that was equivalent to 60 μg/L in the original samples. They were analysed in attenuated order together with pure standards at the same concentration level, and each sample concentration were calculated without IS correction by using the closest standards in the series. The true recoveries were determined on ten samples of each type fortified with ephedrines to levels of 25 and 250 μg/L and with cathinones to levels of 10 and 100 μg/L. The standards used for the determination of the true recoveries were the same blank samples that were fortified after extraction and UF. Finally, the trueness of the method (i.e., the closeness of agreement between the average value obtained from a large series of test results and the accepted reference value) was determined on twenty different blank samples of both live and post-mortem blood that each were fortified with cathinones to levels of 20 and 300 μg/L and ephedrines to levels of 60 and 900 μg/L. These sam-

Table 4
Mass spectrometric conditions and relative retention times (RRTs). The bold and underlined ions were used for quantification. The underlined ions were used as primary qualifiers.

Drug	Transition		Cone voltage (V)	Collision energy (eV)	Relative abundance	RRT
	Q1 (*m*/*z*)	Q3 (*m*/*z*)				
Norephedrine/Cathine	152	**134**/117/91	16	9/16/28	100/25/9	0.29/0.31
Ephedrine/Pseudoephedrine	166	**148**/133/117/91	20	12/20/20/30	100/12/10/8	0.39/0.44
Cathinone	150	132/**117**/105	20	12/22/18	100/40/27	0.40
Flephedrone	182	**149**/103	20	20/27	100/19	0.52
Methcathinone	164	**146**/131/130/105	20	12/20/28/22	100/50/16/14	0.57
Methylephedrine/Methylpseudoeph	180	**162**/147/117/91	24	12/20/20/28	100/20/12/7	0.59/0.66
Ethcathinone	178	**160**/132/105	20	12/18/24	100/48/15	0.70
Methylone	208	**160**/190/132/58	20	18/12/28/13	100/52/36/8	0.76
Methedrone	194	176/**161**/146	20	12/20/28	100/42/20	0.82
Mephedrone	178	**160**/144/119	20	12/25/20	100/18/8	0.84
Butylone	222	**174**/204/191/161	20	18/12/12/18	100/58/22/16	0.93
Amfepramone	206	**105**/133/100/72	28	22/16/22/16	100/78/57/20	1.00

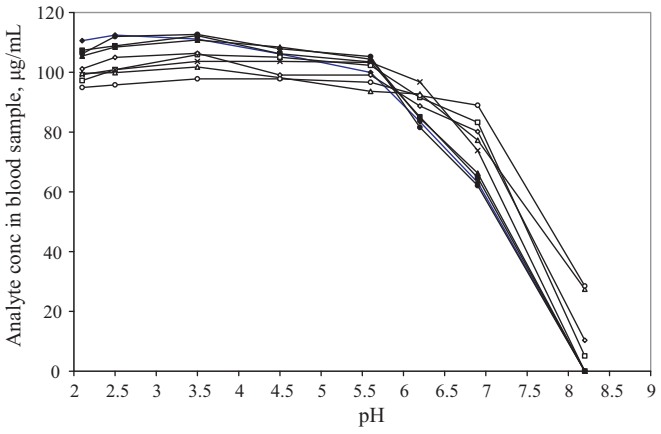


Fig. 1. Stability of cathinone (♦), methcathinone (■), ethcathinone (▲), mephedrone (□), amfepramone (×), flephedrone (●), methedrone (△), methylone (◊) and butylone (○) in the final extracts of live blood fortified with 100 μg/L of each substance and then stored at 20 ± 2 °C for 7 days as function of the pH of the final sample extract.

ples were different from the samples used in the investigation of ion-suppression effects. All eighty trueness samples were analysed according to the procedure.

3. Results and discussion

3.1. Precursor ions and transition products

Electrospray ionisation was achieved in positive ion mode (ESI(+)) for all substances, and the dominant Q1 ions were the protonated molecular ions ($[M+H]^+$). The common fragmentation pattern of ephedrine precursor ions produced $[M+H-H_2O]^+$, *m/z* 91 ($[Ph-CH_2]^+$) and *m/z* 117 ($[Ph-CH=CH-CH_2]^+$) product ions in significant abundance (Table 4). The $[M+H-H_2O]^+$ ions were also produced in significant amounts for cathinones except amfepramone. Furthermore, cathinones with unsubstituted ring structures produced *m/z* 105 ions ($[Ph-C=O]^+$) in significant abundance. For all substances, at least two product ions of acceptable abundance were obtained. The relative abundances of the transition products were constant in the calibrated range, and the mean difference in the relative abundance between the pure reference standards and the matrix-matched calibrants was less than 5% for each substance. The most sensitive transition products were used for the quantitative measurements.

3.2. Extraction and clean-up

The blood samples were extracted by adding MeOH to a final concentration of 70%. The denatured proteins were precipitated by centrifugation, while dissolved high-molecular-weight components were removed by UF through a 30-kDa regenerated cellulose membrane. Before UF, the extract was acidified with formic acid to a pH of 2.5–3 to obtain stable extracts. In non-acidified live-blood extracts, cathinones degraded relatively rapidly. Within the first 24 h of storage at 10 °C, significant reductions in concentrations were observed for flephedrone, amfepramone, ethcathinone and methcathinone. All cathinones except ephedrines were affected by prolonged storage. The instability was clearly pH-dependent (Fig. 1). In the pH range of 2.5–3.5, no significant degradation was observed after a week of storage at 20 ± 2 °C. The UF filtrate was diluted 2-fold with water to reduce the solvent strength before injection on the analytical column.

3.3. Chromatography

Because the ephedrines are found in diastereomeric forms, they cannot be identified solely by mass spectrometry. Chromatographic separation is imperative for the identification of cathine and norephedrine, for example. Sufficient separation of peaks for identification and quantification was achieved for all three pairs of ephedrine isomers using a polymerically bonded phenyl-ethyl phase (Fig. 2). However, the applied chromatographic conditions could not separate flephedrone (4-fluoromethcathinone) from its structural isomer 3-fluoromethcathinone. No detectable carry-over was observed when samples fortified with 1200 μg/L of the individual substances and blank control samples were analysed in attenuated order (four replicate determinations were performed).

3.4. Ion-suppression effects and quantification

To obtain an overview of the variation in ion-suppression effects, the final extracts of 20 randomly selected blank samples each of live blood and post-mortem blood were fortified to a level of 60 mg/L with the different substances and then analysed by LC–MS/MS in attenuated order with respect to sample type. Pure standards were used as calibrants. The data matrix of results, consisting of 40 rows (samples) and 15 columns (substances), was transformed by an row-oriented mean normalization and then treated by principal component analysis (PCA) using cross validation on six random segments. From the score plot of the first two principal components (PCs), it is clear that the ion suppression patterns for live and post-mortem blood are different because the two types of blood samples were separated into two distinct classes (Fig. 3). Thus, accurate quantification is not possible for all analytes using common external matrix-matched calibrants. A comparison of the (PC1, PC2) and (PC2, PC3) score plots reveals that the sample-type differences were explained mainly by PC1. From the loading plot of (PC1, PC2), it could be deduced that norephedrine, ephedrine, cathine, pseudoephedrine and cathinone were the main variables responsible for class separation. Isotope-marked analogues of these substances except cathinone were then included as internal standards in the analytical procedure. According to the PCA, it would also have been feasible to include an analogue of cathinone, but such an analogue was not commercially available at the time of method development. However, cathinone and ephedrine are closely located in the loading plots and an acceptable correction was obtained in practice using ephedrine-D3 as the internal standard for cathinone. The first 3 PCs explained 85% of the variation in the data. To cover the variation between the remaining significant PC's the set of internal standards was extended with the isotope analogues of methcathinone and mephedrone.

In absolute terms, the mean ion-suppression effects were approximately 10, 15, 15, 25 and 40% for pseudoephedrine, ephedrine, cathinone, cathine and norephedrine, respectively. The suppressed substances were characterised by relatively short retention times on the analytical column. For all other substances, the suppression was less than 10%.

3.5. Method performance parameters

The mean true recoveries were better than 87% for both live and post-mortem blood (Table 5). The trueness of the method determined on twenty samples each of live and post-mortem blood samples not included in the method development was close to 100% (Table 6). The LODs of the qualifier ions were in the range of 0.5–3.1 μg/L (Table 7). The RSD_r and $RSD_{R,intra\text{-}lab}$ values obtained in the precision study were not significantly different for the two types of blood samples. The $RSD_{R,intra\text{-}lab}$ values were below 7% for cathinones and 5% for ephedrines at concentrations of 20 and

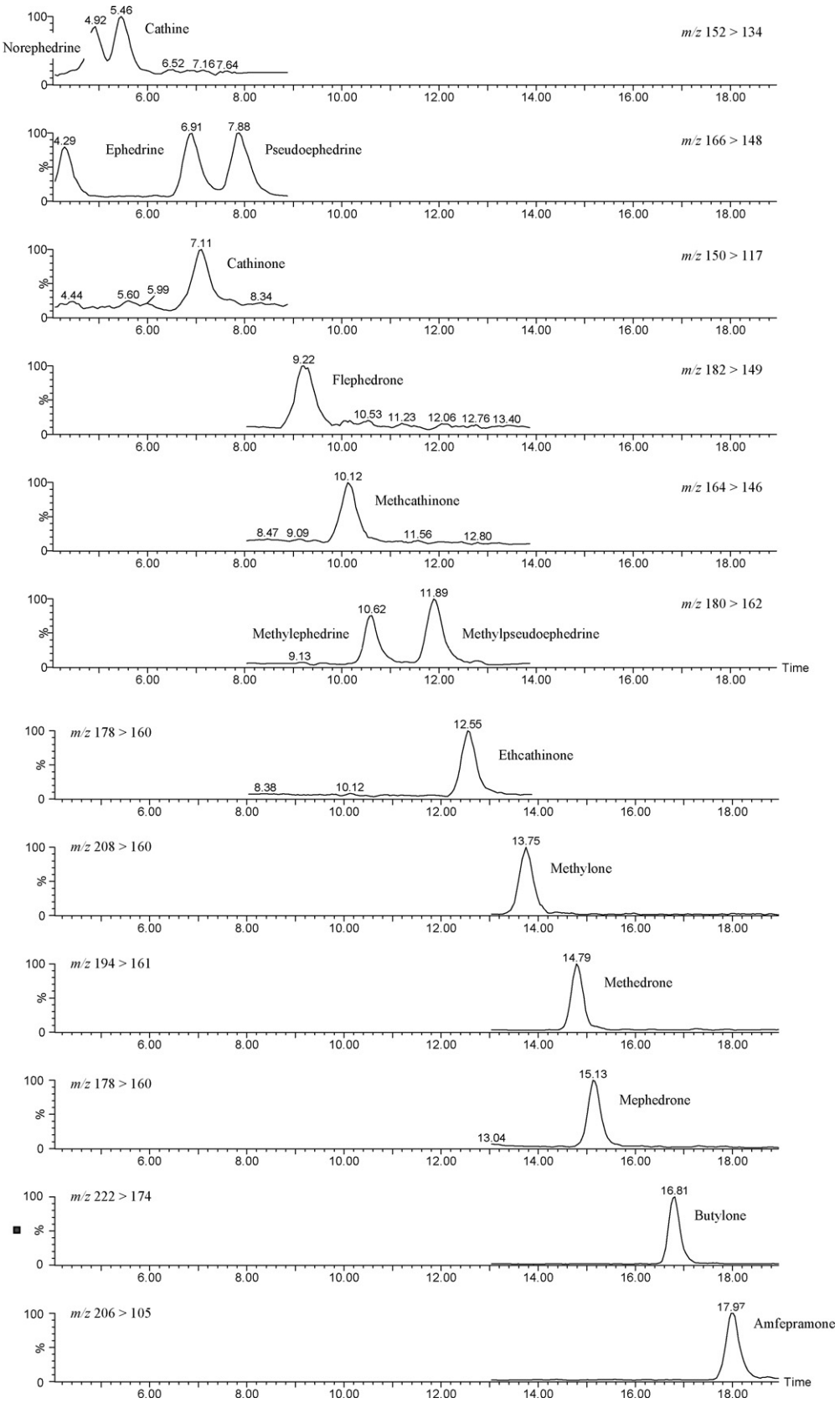


Fig. 2. Chromatograms of the ESI(+) quantifier product ions in the extract of live whole blood fortified with 10 μg/L of each substance. The analytical column was a Prodigy Phenyl-3 (5 μm, 2.0 mm I.D. × 150 mm).

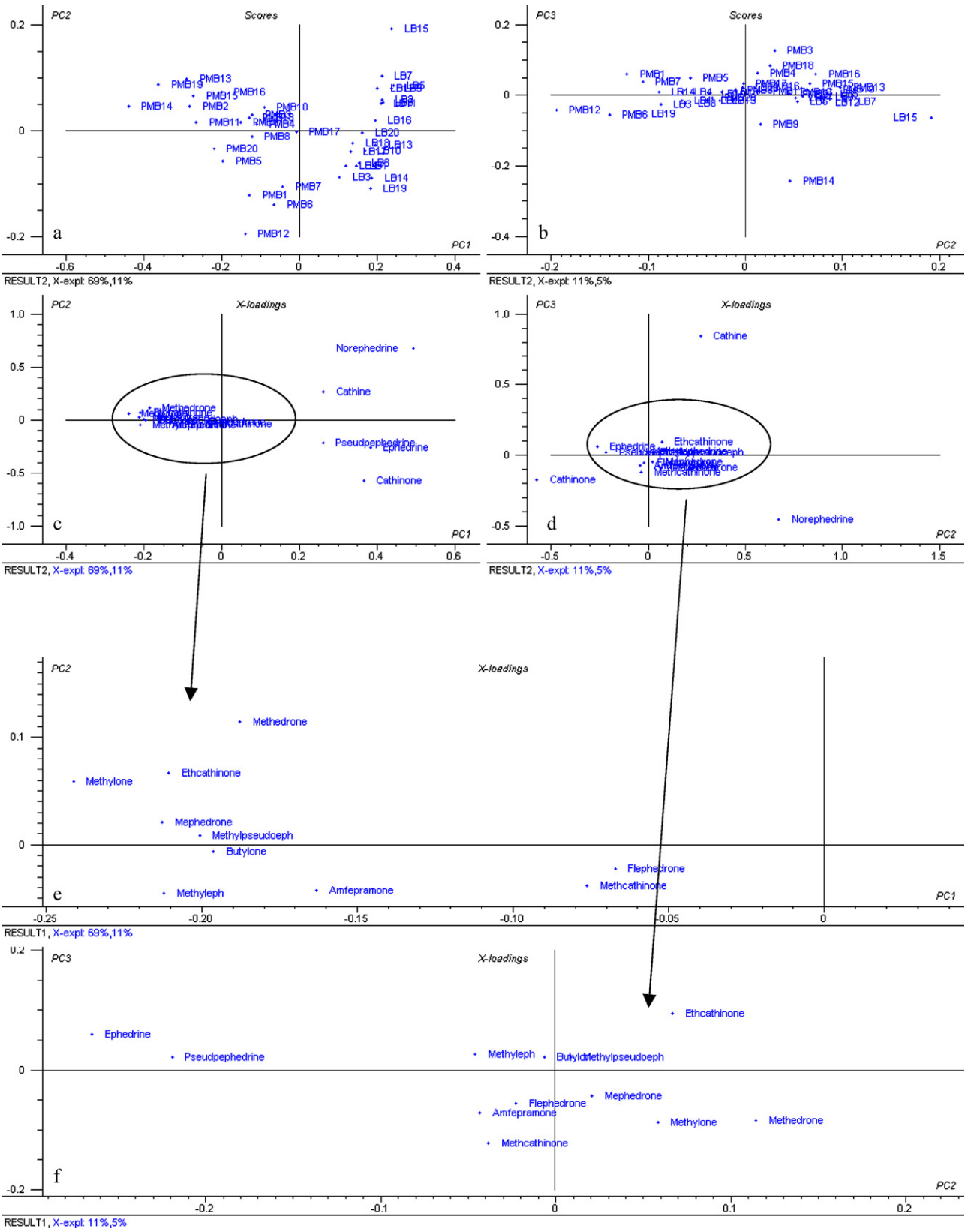


Fig. 3. PCA score and loading plots of fortified sample extracts showing the ion suppression variability between samples. Randomly selected samples of blank live blood (LB, $n = 20$) and post-mortem blood (PMB, $n = 20$) were extracted according to the procedure, and the final extracts were fortified with cathinones and ephedrines to a concentration of 60 μg/L in original sample. The plots (a) and (b) are the score plots of (PC1, PC2) and (PC2, PC3). The plots (c) and (d) are the corresponding loading plots of (PC1, PC2) and (PC2, PC3). The clusters in (c) and (d) are magnified in the loading plots (e) and (f). The first three principal components (PC1, PC2 and PC3) explained 85% of the variation in data.

Table 5
True recoveries obtained from single determinations of fortified live and post-mortem blood samples (n = 10 on each level and for each sample type). Matrix-matched standards used in the calculation of the recovery were the same samples fortified after extraction and UF.

Drug	Fortification level (μg/L)	True recovery, mean (±SD) (%)	
		Live blood	Post-mortem blood
Norephedrine	25/250	87 (±5)/93 (±3)	97 (±9)/98 (±5)
Cathine	25/250	90 (±5)/95 (±3)	96 (±5)/97 (±7)
Ephedrine	25/250	90 (±3)/93 (±3)	100 (±5/100 (±5)
Pseudoephedrine	25/250	96 (±4)/97 (±3)	101 (±4)/100 (±5)
Cathinone	10/100	89 (±3)/94 (±6)	99 (±8)/94 (±6)
Flephedrone	10/100	93 (±9)/94 (±5)	96 (±6)/96 (±5)
Methcathinone	10/100	92 (±4)/97 (±5)	97 (±6)/99 (±4)
Methylephedrine	25/250	101 (±6)/100 (±3)	106 (±4)/103 (±4)
Methylpseudoephedrine	25/250	102 (±8)/100 (±5)	104 (±5)/102 (±5)
Ethcathinone	10/100	96 (±6)/99 (±6)	100 (±5)/101 (±5)
Methylone	10/100	94 (±5)/98 (±5)	102 (±5)/99 (±6)
Methedrone	10/100	93 (±5)/99 (±5)	102 (±7)/100 (±7)
Mephedrone	10/100	96 (±6)/98 (±3)	101 (±5)/101 (±4)
Butylone	10/100	91 (±6)/95 (±3)	100 (±5)/99 (±4)
Amfepramone	10/100	94 (±3)/97 (±4)	102 (±4)/102 (±5)

Table 6
Method trueness obtained from single determinations of fortified live and post-mortem blood samples (n = 20 on each level and for each sample type).

Drug	Fortification level (μg/L)	Trueness, mean (±SD) (%)	
		Live blood	Post-mortem blood
Norephedrine	60/900	97 (±3)/103 (±6)	107 (±6)/100 (±3)
Cathine	60/900	99 (±3)/101 (±6)	100 (±4)/101 (±4)
Ephedrine	60/900	98 (±2)/104 (±3)	103 (±3)/99 (±2)
Pseudoephedrine	60/900	100 (±2)/102 (±2)	101 (±3)/100 (±3)
Cathinone	20/300	101 (±6)/99 (±5)	100 (±6)/100 (±4)
Flephedrone	20/300	101 (±5)/99 (±4)	96 (±8)/100 (±4)
Methcathinone	20/300	100 (±4)/101 (±3)	101 (±4)/101 (±4)
Methylephedrine	60/900	99 (±3)/101 (±2)	102 (±5)/103 (±3)
Methylpseudoephedrine	60/900	99 (±3)/100 (±2)	107 (±6)/102 (±4)
Ethcathinone	20/300	98 (±3)/97 (±3)	98 (±5)/100 (±3)
Methylone	20/300	101 (±3)/100 (±2)	99 (±5)/100 (±3)
Methedrone	20/300	102 (±6)/104 (±2)	101 (±7)/104 (±4)
Mephedrone	20/300	100 (±4)/100 (±4)	100 (±5)/100 (±3)
Butylone	20/300	99 (±3)/99 (±3)	95 (±5)/101 (±4)
Amfepramone	20/300	103 (±4)/97 (±4)	99 (±4)/105 (±3)

60 μg/L, respectively (Tables 8 and 9), which is considered acceptable when compared to the rule of Horwitz [13]. According to the Horwitz equation and practical recommendations [14], an RSD_R of 22% between laboratories would be acceptable at these concentration levels.

The selectivity was investigated by analyzing more than fifty random samples each of live and post-mortem blood that have been tested positive for legal or illicit drugs other than cathinones and ephedrines. In none of these samples transition products of cathinones or ephedrines were observed at the respective retention times with peak areas exceeding the LOD values. More than twenty samples found positive for khat alkaloids by an external laboratory using a different method were also analysed by the present method. No false detections occurred. However, the specificity of the method was not sufficient for the separation of 3-fluromethcathinone and flephedrone (4-fluromethcathinone).

The calibration curves were created using weighted linear regression analysis. The R^2 values obtained in the precision study

Table 7
Limits of detection determined in live and post-mortem whole blood samples (n = 20 of each sample type).

Drug	TransitionQ1/Q3 (m/z)	Fortification level (μg/L)	Result Mean (±SD) (μg/L)		LOD (μg/L)	
			Live	Post m.	Live	Post m.
Norephedrine	152/117	3	4.4 (±0.66)	4.4 (±0.91)	2.0	2.7
Cathine	152/117	3	4.0 (±0.66)	4.2 (±0.74)	2.0	2.2
Ephedrine	166/133	3	3.7 (±0.91)	3.6 (±0.84)	2.7	2.5
Pseudoephedrine	166/133	3	4.0 (±0.96)	3.8 (±1.00)	2.9	3.0
Cathinone	150/105	3	4.1 (±0.69)	3.4 (±1.02)	2.1	3.1
Flephedrone	182/103	3	3.6 (±0.95)	3.8 (±1.03)	2.9	3.1
Methcathinone	164/131	3	3.8 (±0.47)	4.3 (±0.70)	1.4	2.1
Methylephedrine	180/147	3	4.2 (±0.82)	4.0 (±0.76)	2.5	2.3
Methylpseudoephedrine	180/147	3	4.0 (±0.46)	3.9 (±0.53)	1.4	1.6
Ethcathinone	178/132	3	3.6 (±0.52)	4.1 (±0.51)	1.6	1.5
Methylone	208/132	1	0.9 (±0.34)	1.1 (±0.35)	1.0	1.1
Methedrone	194/146	1	0.8 (±0.21)	0.9 (±0.32)	0.6	1.0
Mephedrone	178/144	1	0.9 (±0.23)	1.0 (±0.28)	0.7	0.8
Butylone	222/191	1	1.1 (±0.3)	0.8 (±0.29)	0.9	0.9
Amfepramone	206/133	1	0.9 (±0.18)	1.1 (±0.22)	0.5	0.7

Table 8
Average method precision estimated for cathinones at different drug concentration levels in live and post-mortem whole blood ($n = 1$ for each).

Drug	RSD_r (%) at level			$RSD_{R\ intra\text{-}lab}$ (%) at level		
	4 μg/L	20 μg/L	200 μg/L	4 μg/L	20 μg/L	200 μg/L
Cathinone	9.2	6.2	3.1	12	6.2	4.2
Flephedrone	7.8	4.2	3.1	11	6.0	3.7
Methcathinone	6.0	4.9	2.9	7.7	5.1	3.0
Ethcathinone	6.4	5.5	2.2	11	5.5	3.3
Methylone	7.7	5.3	2.3	7.7	6.1	2.9
Methedrone	6.2	4.7	2.3	8.4	6.3	2.8
Mephedrone	4.8	4.1	2.4	8.1	5.0	3.6
Butylone	7.8	4.1	2.3	7.8	5.4	3.6
Amfepramone	7.9	4.6	2.6	8.5	6.0	3.9

Table 9
Average method precision estimated for ephedrines at different drug concentration levels in live and post-mortem whole blood ($n = 1$ for each).

Drug	RSD_r (%) at level			$RSD_{R\ intra\text{-}lab}$ (%) at level		
	12 μg/L	60 μg/L	600 μg/L	12 μg/L	60 μg/L	600 μg/L
Norephedrine	5.9	4.2	1.3	7.6	4.2	2.3
Cathine	4.5	4.0	1.8	6.1	4.5	2.4
Ephedrine	4.7	4.2	1.3	5.2	4.4	2.6
Pseudoephedrine	5.2	3.7	1.7	7.0	3.7	2.1
Methylephedrine	5.9	4.0	2.3	6.9	4.3	4.9
Methylpseudoephedrine	3.7	3.5	1.8	6.1	3.6	2.2

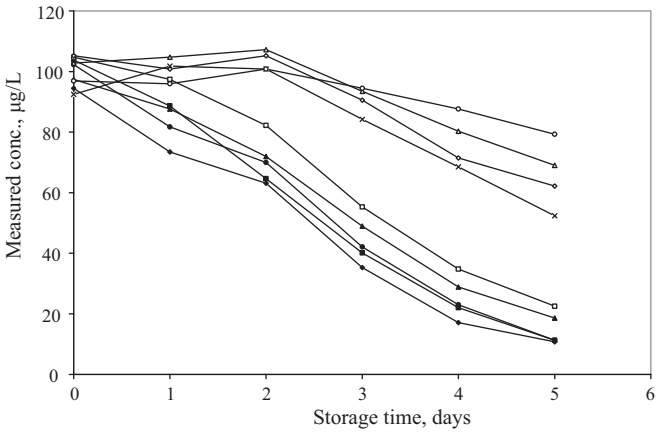


Fig. 4. Stability of cathinone (♦), methcathinone (■), ethcathinone (▲), mephedrone (□), amfepramone (×), flephedrone (●), methedrone (△), methylone (◊) and butylone (○) in live blood ($n = 5$) at a pH of ca. 7.4 when stored at ambient temperature (20 ± 2 °C) in Venosafe tubes containing a fluoride-oxalate additive.

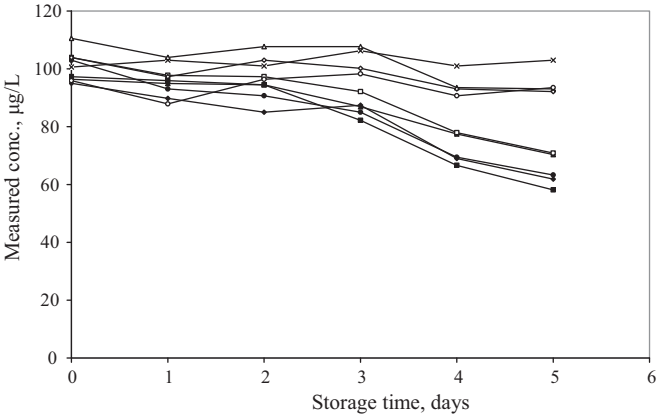


Fig. 5. Stability of cathinone (♦), methcathinone (■), ethcathinone (▲), mephedrone (□), amfepramone (×), flephedrone (●), methedrone (△), methylone (◊) and butylone (○) in live blood ($n = 5$) at a pH of ca. 5.9 when stored at ambient temperature (20 ± 2 °C) in Venosafe tubes containing a fluoride-citrate additive.

from eight independent test series analysed within a 3 weeks period were generally better than 0.997 (overall mean 0.9985, SD 0.0008) for the transitions used in quantification. The mean slope values were in the range 0.0020–0.025 and the RSD values were 1–6% dependent on the substance investigated. The stability of the matrix-matched calibrants at 10 ± 2 °C was tested over a period of 7 days. Calibration curves generated from the absolute peak areas of the stored calibrants were compared with similar calibration curves generated from freshly prepared calibrants that were analysed in the same series. The slopes related to the stored calibrants were within the range of 95–103% of the slopes of the freshly prepared calibrants. The slope differences were not statistically significant.

3.6. Stability of cathinones in blood samples

The stability of cathinones and ephedrines in live blood samples preserved with NaF/potassium oxalate and NaF/citrate buffer, which resulted in sample pHs of ca. 7.4 and 5.9, respectively, was tested at storage temperatures of 20 ± 2 °C and 5 ± 2 °C. The sta-

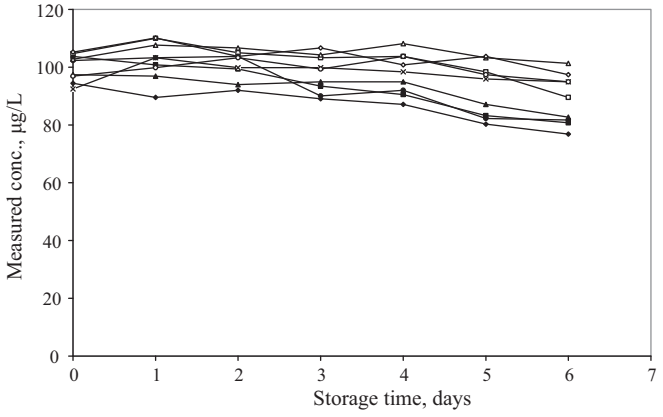


Fig. 6. Stability of cathinone (♦), methcathinone (■), ethcathinone (▲), mephedrone (□), amfepramone (×), flephedrone (●), methedrone (△), methylone (◊) and butylone (○) in live blood ($n = 5$) at a pH of ca. 7.4 when stored at 5 ± 2 °C in Venosafe tubes containing a fluoride-oxalate additive.

bility of cathinones in blood samples was clearly influenced by pH, as in the case of the final extracts. In blood samples preserved with NaF/potassium oxalate, the measured concentrations of cathinone, methcathinone, ethcathinone, mephedrone and flephedrone declined by ca. 30% after 2 days of storage at 20 °C (Fig. 4). When the blood samples were preserved with NaF/citrate buffer, the loss was reduced to ca. 10% (Fig. 5). The other cathinones also decomposed but less rapidly. At a storage temperature of 5 °C, the decomposition proceeded with a markedly lower rate, but a trend was still observed after 3–6 days of storage for samples preserved with NaF/potassium oxalate (Fig. 6). The ephedrines, which contain a hydroxyl group instead of the ketone group at the β position, were stable over the same storage periods regardless of pH and temperature.

4. Conclusion

Cathinones are characterised by instability in blood matrices at neutral and basic conditions. To obtain a robust generic analytical method with high recovery, a simple extraction and clean-up procedure was applied, avoiding critical steps such as solid-phase extraction and solvent evaporation. The ruggedness and reproducibility of the method were secured by use of a properly selected set of isotope-marked analogues as internal standards. The substances included in the method validation were selected cathinones and ephedrines, but the method is probably also applicable for other phenylalkylamines.

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