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# Time-controlled online SPE: strategic approach for N-acylethanolamines quantification in complex matrices

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ABSTRACT

Quantifying and monitoring the level of N-acylethanolamines (NAEs) in biological fluids is becoming increasingly important to better understand their role in health and disease. The complexity of biological matrices, however, poses significant challenges for accurate quantification, with traditional pretreatment methods often proving insufficient in certain cases. This study introduces a novel approach utilizing Online Solid Phase Extraction (SPE) to quantify NAEs in equine plasma, specifically Palmitoyl Ethanolamide (PEA) and Oleoyl Ethanolamide (OEA). In the original analytical method here developed and validated, established toluene liquid-liquid extraction was used to isolate the lipid-like fraction, followed by an innovative Time-Controlled Online SPE coupled to HPLC-MS/MS. This strategic temporal approach allows target analytes to rapidly elute to the analytical column while retaining lipidic interferents, preventing matrix contamination and ensuring selective analysis. The method validation demonstrated excellent linearity, while providing high recovery and suitable matrix effects. The limits of detection (LOD) and quantification (LOQ) were determined as 0.27 ng/mL and 0.83 ng/mL for PEA; 0.04 ng/mL and 0.11 ng/mL for OEA. The innovative approach here presented allowed for reliable NAE quantification in equine plasma and provided unprecedented data on the endogenous levels of PEA and OEA in this species. This method has potential applications for the analysis of other complex biological matrices with high levels of interferents that share chemical similarities with the target compounds. This novel approach significantly advances the understanding of the endocannabinoid system in equines and serves as a valuable tool for future research in this domain.

## 1. Introduction

Endocannabinoids and related N-acylethanolamines (NAEs) are vital signaling molecules regulating a variety of bodily functions, like metabolism, immunity and pain processing [1]. Changed levels of endocannabinoids and NAEs have been linked to a number of disorders, including chronic pain, neurodegeneration and inflammatory diseases [2–4]. Modern analytical techniques [5], like high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), have significantly improved our ability to measure these compounds in different tissues and body fluids [6]. This has allowed us to improve our understanding of their role in health and disease, thanks to studies carried out on samples from different mammalian species [7]. Although blood is one of the easiest biological samples to obtain, matrix effects may occur and impact the trueness of HPLC/MS/MS results. Therefore, pretreatment is crucial for the analysis of NAEs in plasma samples [8]. Liquid-liquid extraction (LLE) is the most widely employed pretreatment technique, due to its simplicity and effectiveness; selection of the solvent is, however, fundamental. Although acetonitrile and methyl tertiary-butyl ether (MTBE) are frequently employed [6], toluene is regarded as the most effective solvent for the treatment of plasma samples [9]. A range of extraction methodologies have been developed for the quantitative determination of NAEs in human and murine plasma [10,11]. Unfortunately, LLE is not always appropriate for complex biological samples, due to matrix interferents, so the extraction with organic solvent should be followed by a second extraction procedure. This step

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could be carried out with Solid Phase Extraction (SPE) which is considered one of the most effective methods [12–15]. Different SPE methods, including micro-SPE [16,17], magnetic SPE [18], dispersive SPE [19] and Online SPE [20], have been developed and are widely used in the pharmaceutical [21,22], environmental [23], and alimentary fields [24]. Online SPE has several advantages and can be seamlessly integrated into the HPLC line, allowing to manage both sample preparation and analysis within a unified continuous process [18,25]. However, conventional online SPE approaches for lipophilic analytes typically rely on sequential washing and elution phases: hydrophilic mobile phases first eliminate polar interferents, followed by organic solvents for analyte elution. This approach becomes ineffective when both target compounds and matrix interferents share similar chemical properties, as occurs in lipid-rich biological matrices. Online SPE has been recently used coupled to ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC—HRMS) for the simultaneous determination of a broad range of PerFluorinated Alkylated Substances (PFAS) in human serum [26]. To the best of our knowledge, N-acylethanolamines (NAEs) in equine plasma have not been the subject of exploration. A more comprehensive understanding of these compounds could offer significant insights into the metabolic processes of horses [27]. The composition and functional properties of this biological matrix are determined by the presence of phospholipids, proteins, organic acids, and wide spectrum of additional compounds [28–30]. Traditional and online solid-phase extraction (SPE) protocols rely on chemical selectivity through washing phases designed to eliminate interferents with distinct chemical behaviour from target analytes. However, this approach becomes ineffective when interferents possess a similar affinity to the stationary phase as the analytes themselves. Our breakthrough, which is controlled by time, addresses this critical limitation bypassing conventional washing protocols and initiating separation directly with the eluting phase. This establishes temporal discrimination as the decisive purification mechanism for chemically indistinguishable compounds. In this context, temporal control emerges as a critical parameter: by precisely timing the elution and retention phases, it becomes possible to separate chemically similar compounds based on their differential retention times rather than relying solely on chemical selectivity. Here we successfully developed and validated an innovative time-controlled online SPE configuration coupled with HPLC-MS/MS, that allowed for the quantitative analysis of two NAEs in equine plasma, Palmitoyl Ethanol Amide (PEA) and Oleoyl Ethanol Amide (OEA). This approach exploits strategic timing to allow rapid analyte elution while retaining matrix interferents, which are subsequently eliminated through a parallel washing cycle. In order to accomplish this aim, a detailed examination of the elution times of the analytes of interest was essential. Subsequently, the SPE column was properly installed on the HPLC line to achieve the desired configuration. By appropriately regulating the switching times of the six-way valve, it is possible to recover only the analyte and ensure that the interferents do not reach the analytical column. This approach prevents unwanted accumulation, which would significantly interfere with the quantitative analysis. This strategy has been shown to enable a substantial reduction in the concentration of interferents, allowing HPLC-MS/MS analysis to be performed on cleaner samples.

## 2. Materials

### 2.1. Chemicals and reagent

Methanol grade LC/MS, Water grade LC/MS, Tert-butylmethylether (TBME) grade HPLC, Acetonitrile grade LC/MS, Toluene were purchased from Carlo Erba Reagents (Milan, Italy). Argo Lab Mix Vortex and refrigerated micro centrifuge DLAB D1524R were used to homogenise the samples. The solid phase extraction was performed with an Oasis HLB Online Column, 80 Å, 15 µm, 4.6 mm X 20 mm, 1/pk supplied by Waters (Framingham, Massachusetts, U.SA.) The following standards were purchased from Cayman Chemical Company (Ann Arbor, Michigan):Palmitoyl Ethanol Amide 13C (PEA-13C or N-(2-hydroxyethyl)-hexadecanamide-1,2,3,4–13C), Palmitoyl Ethanol Amide (PEA or N-(2-hydroxyethyl)-hexadecanamide), Oleoyl Ethanol Amide d4 (OEA-d4 or N-(2-hydroxyethyl-1,1,2,2-d4)-9Z-octadecenamide), Oleoyl Ethanol Amide (OEA or N-(2-hydroxyethyl)-9Z-octadecenamide).

### 2.2. Biological samples

Thirty-six biological samples were collected from healthy show-jumping horses. After collection, the blood was placed into plastic tubes with EDTA and stored in ice for 4 min. Samples were then centrifuged at 2000 rcf for 5 min, then aliquoted into 5 Eppendorf tubes and finally stored at −80 °C until analysis.

## 3. Methods

### 3.1. HPLC-MS/MS conditions

The HPLC–MS/MS system consisted of Thermo Scientific Vanquish HPLC and Thermo Scientific LTQ XL, Thermo Scientific Ion Max H-ESI. Thermo Xcalibur Data Acquisition and Processing Software Version 4.2® was used for data acquisition and processing. The column (Kinetex 1.7 µm EVO C18 100 × 2.1 mm; Phenomenex, Aschaffenburg, Germany) was equipped with security guard columns (C18, 4 mm × 2.0 mm; Phenomenex). The mobile phase consisted of (i) water (20 %), (ii) acetonitrile (50 %), and (iii) methanol (30 %), each containing 0.1 % formic acid. The elution was carried out isocratically. The column temperature was set at 40 °C, and the injection volume was 20 µL. The MS instrument settings for maximum signal were optimized with compound quantitative optimization wizard in the Tune Plus®. The single reaction monitoring (SRM) and transitions are presented in Table 1, two precursor/product ions (Q1/Q3) were monitored for the selected analyte. The principal operational parameters of the mass spectrometer were as follows: curtain gas (CUR) 25, collision gas (CAD) 28–33 ua, ion spray voltage 2500 V, ion source gas 1 (GS1) 60 psi, temperature 350 °C and resolution unity.

### 3.2. Setup and time-controlled of online SPE

To implement the HPLC-MS/MS method and overcome the issues related to matrix interference, an Online SPE column was mounted on the HPLC line exploiting temporal control to take advantage of the differential elution times of analytes and interferents in the chromatographic system. In this method, target NAEs elute earlier than lipidic matrix interferents, enabling the timing strategy to be designed to selectively transfer only the analytes to the analytical column: during the optimized time window (timing optimization described in Section 4.2), fluidic configuration was in 1_2 position and the analytes of interest (i.e., NAEs) were eluted and sent to the analytical column (Fig. 1); thereafter the system was programmed to automatically switch the valve fluidics to position 6_1, preventing later-eluting interferents from reaching the analytical column and directing the retained interferents from the Online SPE to waste (Fig. 2). During the last minute of the analysis the system returned to the initial configuration to re-balance the Online SPE column and prepare for the next analysis.

The programmed chromatographic run had a total duration of 15 min and both HPLC pumps were used. The left one was dedicated to the analytical column, with the mobile phase (A: water (20 %), B: acetonitrile (50 %), and C: methanol (30 %)) having a flow of 0.3 mL/min; while the right one was dedicated to the Online SPE line with a flow of 0.2 mL/min. During the first 4 min the mobile phase was the same and changed thereafter until minute 9.54 when the percentage of solvents changed for the washing phase change to 100 % methanol at 0.3 mL/min to eliminate part of the interferent retained matrices.

**Table 1**
Qualificator (Q1) and Quantifier (Q3) ions, Calibration curves equation, determination coefficient ($R^2$), LOD and LOQ for the study analytes.

| | Type | Q1 (Da) | Q3 (Da) | Linearity | $R^2$ | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|---|---|
| **PEA** | **STD** | 300.2 | 283.3 | $y = 0.052+0.112*x$ | 0.9994 | 0.27 | 0.83 |
| **PEA $^{13}$C4** | **IS** | 304.4 | 287.3 | | | | |
| **OEA** | **STD** | 326.3 | 308.4 | $y=-0.004+0.111*x$ | 0.9995 | 0.04 | 0.11 |
| **OEA D4** | **IS** | 330.4 | 312.4 | | | | |

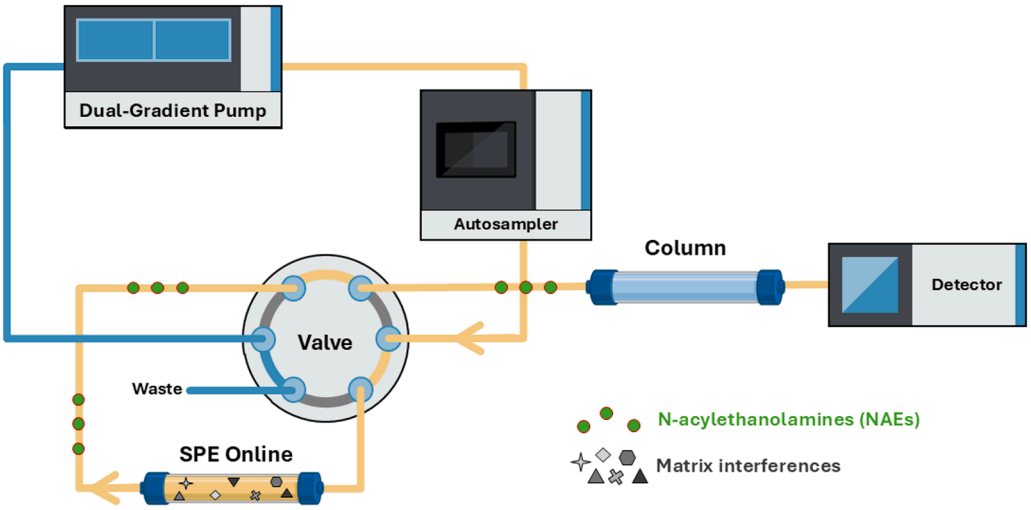


**Fig. 1.** Configuration 1_2 of the online SPE and valve switching system.

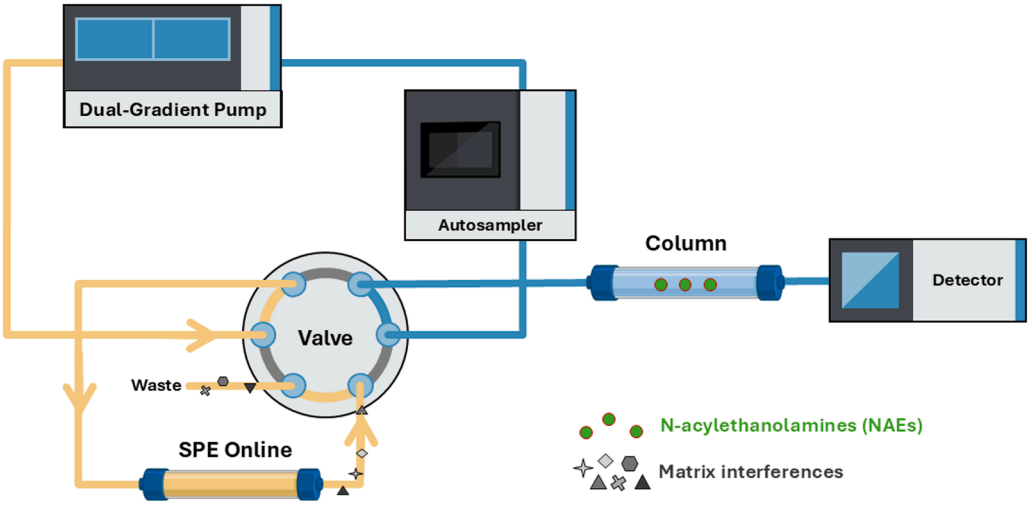


**Fig. 2.** Configuration 6_1 of the online SPE and valve switching system.

### *3.3. Sample extraction methods*

The toluene-based LLE protocol was selected after preliminary evaluation of three different extraction procedures (But/MeOH [31], TBME [6] and Toluene[9]) reported in literature for NAE analysis. Among the tested approaches, toluene extraction provided the highest recovery and cleanest extracts for the target analytes in equine plasma. The toluene-based LLE protocol was selected after preliminary evaluation of three different extraction procedures (But/MeOH [31], TBME [6] and Toluene [9]) reported in literature for NAE analysis. Detailed comparative data including recovery rates, matrix effects, and chromatographic cleanliness for all three extraction methods are provided in SI, Table S2. Among the tested approaches, toluene extraction provided the highest recovery and cleanest extracts for the target analytes in equine plasma. Since LLE optimization was not the primary focus of this study, the established toluene method was adopted to ensure reliable pre-purification of the lipid-like fraction before implementing the innovative time-controlled online SPE approach. Biological samples were pre-treated by liquid/liquid extraction (LLE), by adding 150 µL of toluene solution containing the internal standards (IS) (100 ng/mL) to 50 µL equine plasma. Samples were homogenised by vortexing for 30 s and then centrifuged for 5 min at 4 °C and 12.000 x g. Finally, the supernatant was collected, transferred to a clean Eppendorf tube and evaporated to dryness under a stream of nitrogen. The last step required the sample to be reconstituted with 200 µL acetonitrile (ACN) and transferred to a 0.2 mL glass insert with a Teflon stopper for analysis by

HPLC ESI-MS/MS (Fig. 3).

### 3.4. Method validation

The methods were validated based on the ICH (International Council for Harmonisation), Q2-R2 and M10 on Bioanalytical Method Validation parameters [32].

#### 3.4.1. Linearity range

Calibration curves were constructed from data obtained from the analysis of samples with known concentrations (seven levels of agreement). The IS concentration employed was 10 ng/mL, while the concentration range of the standards for the method spanned 0.5 to 25 ng/mL (0.5, 1, 2, 5, 10, 15, 25 ng/mL). Calibration linearity curves were obtained by plotting the peak area ratios obtained for single analyte and related IS against the nominal concentration. The goodness of fit was assessed by the linear determination coefficient $R^2$. Curves were plotted in triplicate and standard deviation calculated for slope and intercept.

#### 3.4.2. LOD and LOQ

The limit of quantification (LOQ) was established as the lowest concentration at which the variability of trueness and precision ( %CV) was less than 20 %, and the signal-to-noise ratio was greater than 10, determined by injecting serially diluted calibrators. The limit of detection (LOD) was defined as the concentration of the analyte that produced a signal significantly different from the blank. This was the concentration at which the signal-to-noise ratio was greater than 3.3. LOD and LOQ values were calculated using the standard deviations ($\sigma$) of the y-intercepts and the slopes of the regression lines (S) from the standard curves for each analyte and respectively expressed as $3.3\sigma/S$ and $10\sigma/S$.

#### 3.4.3. Trueness

The trueness of the analytical method was determined by estimating the recovery (R) and the matrix effect (ME). The recovery value was calculated as $R\ (\%) = A/B \times 100$, where A is the value obtained from the analysis of the sample fortified with all standards (including IS) and subjected to extraction, while B is the value obtained from the analysis of the sample subjected to extraction and subsequently fortified with all standards. The recovery was measured from quality control (QC) samples at three different concentrations: low (LQC), medium (MQC), and high (HQC), respectively 7, 10 and 20 ng/mL.

The ME was assessed by matrix factor determination (MF, %), calculated as $(C—C_e)/D) \times 100$, where C is the analyte peak area of the biological sample spiked with the standard sample after extraction, $C_e$ is the analyte peak area of the biological sample after extraction and D is the analyte peak area of the pure standard sample in the absence of the biological matrix. The measurements were carried out with additions of standard samples at three different concentrations: low (LQC), medium (MQC), and high (HQC), respectively 7, 10, 20 ng/mL.

#### 3.4.4. Precision

Precision, expressed as intra-day and inter-day variation, was estimated by determining the coefficient of variability ( %CV) in three replicates. Precision batches consist of quality control (QC) samples at three different concentrations (low quality control (LQC), middle quality control (MQC) and high-quality control (HQC)) for all tested compounds, respectively 7, 12, 20 ng/mL.

#### 3.4.5. Carryover

Carryover (defined as the highest analysable concentration for which no significant analyte signals are observed in the subsequent blank) was assessed by recording the signal from the highest calibration concentration (25 ng/mL) and then running a blank. The analyte signal in the blank had not to exceed 10 % of the signal from the preceding standard.

#### 3.4.6. Statistical analysis

The Dixon test, also known as Dixon's Q test, was used to identify the outliers. Data set for PEA and OEA concentrations was arranged in ascending order. The $Q_n$ ratio was then calculated according to Eq. (1), where R is the difference between the largest and smallest values in the

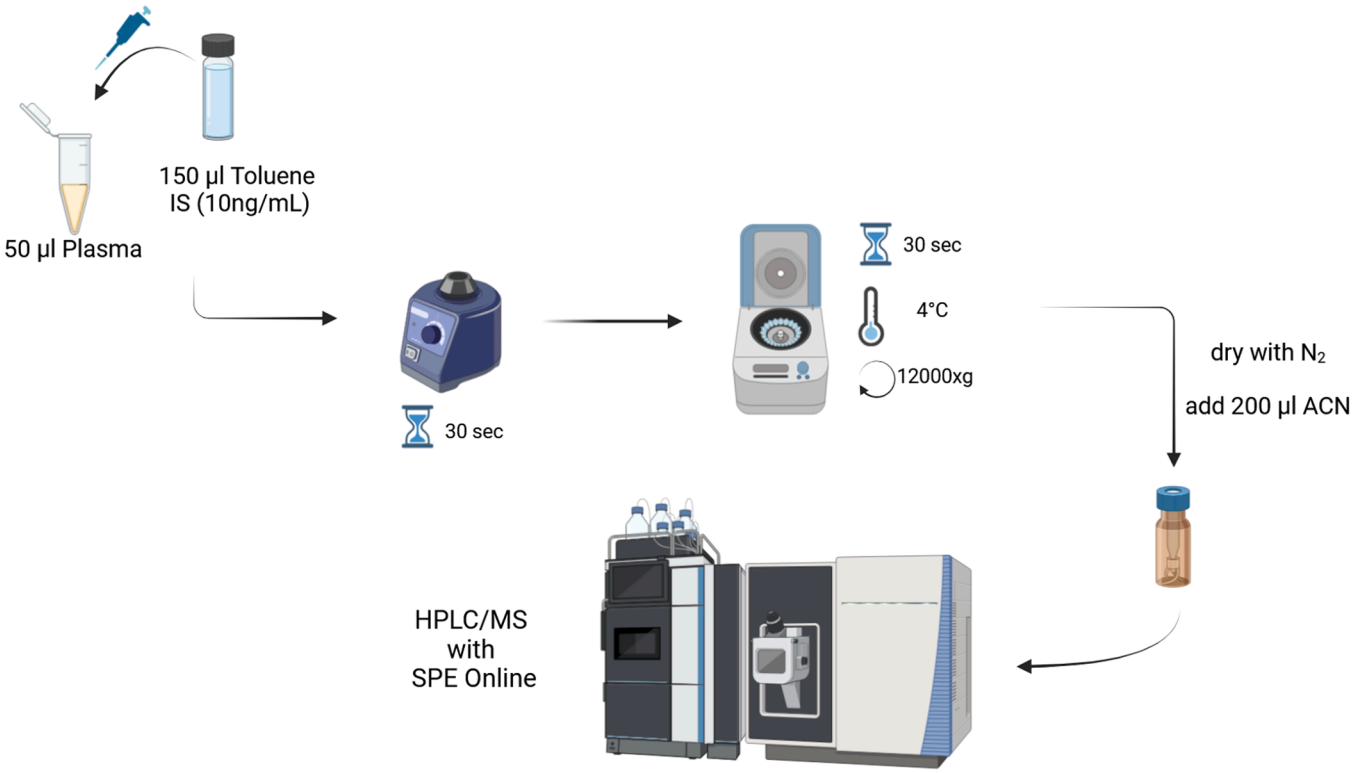


**Fig. 3.** Liquid/liquid extraction (LLE) procedure and analysis with HPLC ESI-MS/MS.

data set and $x_n$ is the alleged outlier.

$$Q_n = \frac{|x_n - x_{n-1}|}{|R|} \quad (1)$$

The calculated $Q_n$ value was then compared to a tabulated critical value, based on the chosen significance level (α) and the number of samples. If $Q_n >$ critical Q, the value was identified as an outlier [33,34].

## 4. Results and discussion

Here we successfully applied a new HPLC–MS/MS-coupled online SPE with a Time-Controlled configuration to the quantitative analysis of two NAEs in equine plasma, namely PEA and OEA.

Conventional online SPE approaches could not be employed in our case due to the chemical similarity between analytes and interferents. Traditional online SPE methods for lipophilic compounds typically use hydrophilic washing phases to eliminate polar interferents before organic analyte elution. However, both NAEs and plasma interferents (phospholipids, lipoproteins) exhibit similar lipophilic characteristics, making conventional hydrophilic washing ineffective. Therefore, toluene liquid-liquid extraction was essential to pre-select the lipid-like fraction, followed by our dual-line approach: the first line transfers analytes to the analytical column while the second operates in parallel to wash retained interferents with methanol. This temporal separation strategy overcomes the chemical similarity limitations of conventional protocols.

### 4.1. Method validation

#### 4.1.1. Linearity range and limits (LOD and LOQ)

The goodness of fit, as assessed by the Linear Determination Coefficient $R^2$, ranged from 0.9994 to 0.9997 for PEA and from 0.9992 to 0.9995 for OEA, indicating that the method is linear over the selected calibration range. The standard deviation was calculated for the intercept (0.009 for PEA and 0.001 for OEA) and for the slope (0.004 for PEA and 0.003 for OEA). These data were obtained from the curves plotted in triplicate and reported in the supporting information section. The analyte response for the quantifier transition was normalized to IS and calibration was achieved by weighted linear least squares regression (weight 1/x2), the calibration straight lines and the relative $R^2$, are shown in Table 1. The limits of detection (LOD) and quantification (LOQ) were found to be similar to those reported for methods already validated for plasma matrices [5].

#### 4.1.2. Trueness

The trueness of the analytical method was determined by estimating the recovery (R) and the matrix effect (ME). The results are detailed in Table 2.

#### 4.1.3. Precision

The precision values for detection of PEA and OEA in equine plasma samples are detailed in Table 3. The results were expressed as intra-day and inter-day coefficients of variation (CVs) and were below 6 %, which is within the acceptable range (CV < 15 %) [32].

#### 4.1.4. Carryover

Carryover effect was evaluated with the blank samples following up the highest calibrator running and found to be less than 10 % of the signal of analytes at LOQ for all the study analytes. No carry over (signal < LOD) was observed after injection of the control standard (CS) at the upper limit of quantification.

**Table 2**
Trueness values expressed as Recovery and Matrix effect for all analytes.

| | Recovery (%) | | Matrix effect (%) | |
|---|---|---|---|---|
| Concentration | PEA | OEA | PEA | OEA |
| LQC (7 ng/mL) | 89.89 | 104.20 | 92.38 | 84.82 |
| MCQ (10 ng/mL) | 103.35 | 108.58 | 92.71 | 84.70 |
| HCQ (20 ng/mL) | 104.36 | 113.75 | 92.92 | 87.77 |

**Table 3**
Precision values expressed as coefficient of variability (CVs) for all analytes.

| | Intra-day %CV | | Inter-day %CV | |
|---|---|---|---|---|
| Concentration | PEA | OEA | PEA | OEA |
| LQC (7 ng/mL) | 3.84 | 5.04 | 0.69 | 3.37 |
| MCQ (12 ng/mL) | 0.29 | 2.89 | 1.68 | 3.43 |
| HCQ (20 ng/mL) | 3.92 | 3.42 | 2.31 | 1.83 |

#### 4.1.5. Statistical analysis - outliers

Few values of the quantitative analysis of OEA were identified as possible outliers and confirmed as such at the Dixon test (assuming a critical Q with a confidence level of 99 % for a set of samples greater than 10). The identified outliers were discarded accordingly.

### 4.2. Optimization of six-way valve switching time

To determine the optimal switching time for the six-way valve in the online SPE configuration, systematic experiments were conducted by varying the valve switching time at different intervals. Three representative switching times (5, 4, and 3 min) were selected as the most informative for demonstrating the critical impact of timing on analyte recovery and matrix interference elimination.

When the valve was switched at 5 min (Fig. 4a), both analytes (PEA shown as red line, OEA shown as green line, and their respective internal standards) were successfully transferred to the analytical column. However, this configuration also allowed matrix interferents, primarily lipid-like materials including phospholipids (black line), to reach the analytical column, resulting in interferent accumulation on the column that requires extensive subsequent washing cycles and compromised overall analytical performance.

The optimal switching time was identified at 4 min (Fig. 4b), where all target analytes were efficiently eluted to the analytical column while matrix interferents (black line) were retained on the SPE column. This configuration achieved the desired separation between analytes and interfering compounds, ensuring clean chromatographic analysis without compromising analyte recovery. The red line (PEA) and green line (OEA) remained clearly detectable with optimal peak areas.

When the valve switching was performed earlier at 3 min (Fig. 4c), one of the target analytes, OEA (green line, retention time 6.06 min), was not transferred to the analytical column and disappeared from the chromatogram. Additionally, the peak area of PEA (red line) was significantly reduced, indicating partial analyte loss due to premature switching. This demonstrated that the elution window must be carefully optimized to ensure complete recovery of all target compounds while maintaining effective matrix cleanup. Multiple switching times between 3 and 4 min were tested (3.25, 3.5, and 3.8 min), and additional chromatographic data reported in Supporting Information (Fig. S1, S2 and S3) demonstrate that the 4-minute switching time achieves a 2 orders of magnitude reduction in interferent Total Ion Current (TIC) compared to longer switching times, while individual analyte overlays show progressive analyte loss between 3 and 4 min switching times. Specifically, OEA showed peak area at 3.25 min (black line) reduced to 50 % and at 3.5 min (red line) to 80 % compared to the 3.8 min condition (green line), which provided near-optimal analyte transfer. In contrast, PEA displayed only ~10 % variation in peak area across the tested switching times, with optimal analyte transfer for both compounds maintained at the 4-minute switching condition. The 4-minute switching time consistently provided the best results in terms of analyte recovery and matrix interference elimination.

Based on these systematic optimization studies, the 4-minute

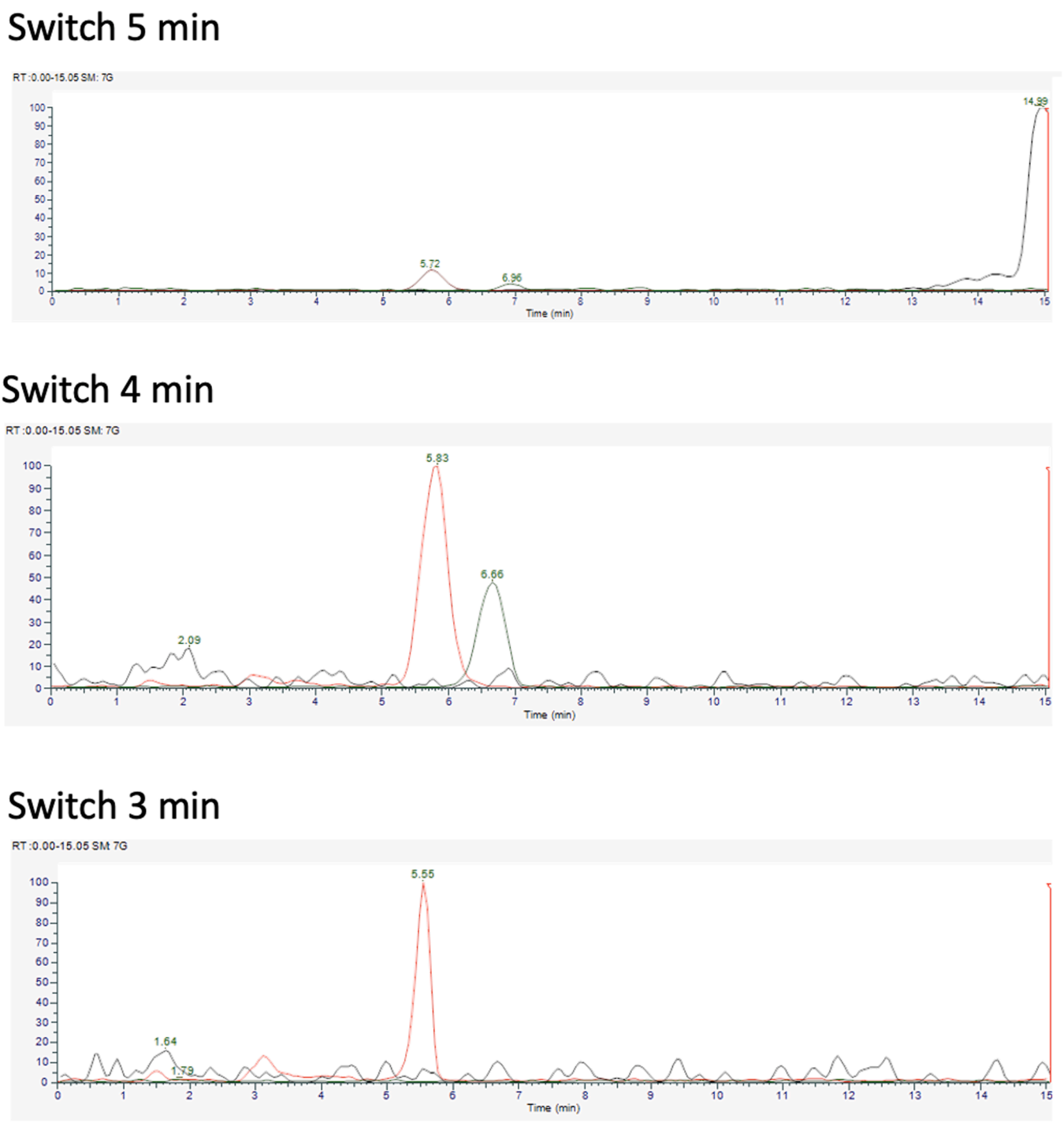


**Fig. 4.** Effect of valve switching time on analyte recovery and matrix interference. (a) 5-minute switching; (b) 4-minute switching; (c) 3-minute switching. PEA-red line,OEA-green line, matrix interferents-black line.

switching time was selected for the final analytical method, as it provided the optimal balance between complete analyte recovery and effective matrix interference elimination. After the valve switching, a separate solvent line delivers methanol to the SPE purification column to wash away the retained interferents and regenerate the online SPE column for subsequent analyses, without sending any of this wash solution to the analytical column.

### *4.3. SPE strategy performance*

#### *4.3.1. Conventional SPE assessment*

We performed the offline solid-phase extraction (SPE) comparison using two approaches: 1) direct plasma loading after filtration (Fig. S3a), and 2) reconstitution of dried toluene extracts in an aqueous medium (Fig. S3b). Direct plasma analysis using conventional SPE protocols involving hydrophilic washing phases was unsuccessful in removing lipidic interferents chemically similar to NAEs. Since the hydrophilic interferents had already been eliminated during the liquid-liquid extraction stage, the remaining analytical challenge was to separate

the lipophilic analytes from the analogous matrix components (e.g. phospholipids, etc.…). Both experimental approaches yielded unsatisfactory results, confirming that traditional SPE selectivity mechanisms cannot effectively discriminate between compounds with comparable chemical properties. These results validate the rationale behind our methodological development and confirm the necessity of the time-controlled approach.

#### 4.3.2. Extraction results with and without time-controlled online SPE

The conventional SPE turned out to be unsuitable for equine plasma samples due to chemical similarity between the interferent lipids and the target analytes.

For comparison purposes, plasma samples were analysed both with and without online SPE in the reverse configuration mode. In particular, different aliquots of each sample were subjected to LLE with toluene and analysed in triplicate by both HPLC ESI-MS/MS and reverse mode online SPE into the system. Plasma samples analysed without reverse online SPE exhibited significant variation in the elution times of the internal standards (IS) and the chromatographic peak area. Moreover, a high standard deviation was observed. These variations observed in the same sample can be considered a direct consequence of matrix interferents accumulating in the chromatographic column and increasingly influencing the subsequent analysis. On the contrary, samples analysed with online SPE (in the reverse mode configuration) showed no variation in the elution times or recorded peak areas. Moreover, the measured levels of the analytes showed that the unconventional online SPE effectively mitigated interference-related issues. We thus applied the newly developed method to determine the levels of two NAEs, PEA and OEA, in the equine plasma. Although several studies have investigated the plasma level of NAE in healthy and diseased human beings [35–37] and privately-owned dogs [38,39] no data are yet available in the equine subjects. Thirty-six samples from healthy showjumping horses were analysed for the quantification of basal plasma levels of PEA and OEA. With reference to OEA, two samples were eliminated due to the levels falling below the LOQ and two additional samples were identified as outliers at the Dixon test. Consequently, 32 samples were considered for OEA measurement. The results are summarized in Table 4. The reported concentrations were calculated applying the appropriate dilution factors from the sample preparation procedure, confirming that all measurements fell within the validated calibration range and ensuring the reliability of the reported values. The high standard deviation is due to the intrinsic interindividual variability in the levels of NAEs and is consistent with findings recently reviewed in human beings [37] and observed in privately-owned dogs [38,39]. The mean plasma levels of PEA in equine subjects were hundreds of times higher, compared to those previously found in plasma from healthy human and canine subjects, which ranged from 0.8 nmol/L to 10.5 nmol/L [37] and from 28.4 nmol/L to 35 nmol/L[38,39], respectively. On the other hand, the mean plasma levels of OEA in horses was dozens of times higher compared to human healthy controls (0.3–7.5 mol/L)[37] and lower compared to healthy dogs (20–56 nmol/L) [38,39]. These discrepancies may reflect species-specific differences, although methodological issues cannot be ruled out.

In conclusion, this study confirms that liquid-liquid extraction with toluene remains the method of choice for NAE isolation [40] given its high recovery and minimal matrix interference. Nonetheless, the unique composition of equine plasma renders liquid-liquid extraction alone insufficient, requiring additional purification steps. Our trials with conventional SPE yielded inadequate results in this matrix, adversely affecting overall analytical performance. To overcome these limitations, an alternative online SPE configuration is adopted as a complementary step to LLE. Moreover, integrating a tailored "hyphenated" technique pre-treatment between LLE and the alternative online SPE proved instrumental in effectively neutralizing matrix-related interferences. This refined strategy ultimately renders the method validatable for the quantification of key biomarkers such as PEA and OEA in equine plasma.

**Table 4**
Result from quantitative determination of PEA and OEA on equine plasma samples, expressed as mean ± standard deviation (nmol/L).

| PEA (nmol/L) | OEA (nmol/L) |
|---|---|
| 238.29 ± 70.10 | 6.74 ± 3.77 |

## 5. Conclusions

This study presents the first application of time-controlled online SPE, where valve switching timing is strategically optimized to achieve temporal separation of chemically similar compounds. The innovative approach demonstrates that precise temporal control can overcome analytical challenges that conventional SPE methods cannot address, particularly when target analytes and matrix interferents share similar physicochemical properties.

The time-controlled configuration enabled selective analyte elution while retaining lipidic interferents, preventing column contamination and ensuring analytical reliability. Through systematic optimization of switching timing, we identified the optimal temporal window that provides the best balance between complete analyte recovery and effective matrix cleanup. This temporal separation strategy represents a paradigm shift from traditional chemical selectivity-based separations to time-dependent purification protocols.

The method underwent comprehensive validation, with all analytical parameters meeting established criteria for bioanalytical methods. The successful application to equine plasma, a particularly challenging lipid-rich matrix, resulted in the first quantitative determination of PEA and OEA levels in this species (238.30 nmol/L and 6.73 nmol/L, respectively).

The time-controlled online SPE approach addresses a fundamental limitation in analytical chemistry: the purification of target compounds from matrices containing chemically similar interferents. This methodology has broad applicability beyond equine plasma, offering solutions for other complex biological matrices where conventional purification strategies fail. The temporal control principle could be extended to diverse analytical challenges in pharmaceutical, environmental, and clinical analysis, where matrix complexity compromises analytical performance. The method could be useful in the domain of equine anti-doping research providing a valuable tool for monitoring endogenous biomarkers that may be altered by prohibited substances. Likewise, for the monitoring of inflammatory conditions, where NAEs are known to play a crucial role, their accurate quantification is fundamental to understanding the complex pathophysiology of these processes.

## CRediT authorship contribution statement

**Nicoletta Gatti:** Writing – original draft, Validation, Formal analysis, Data curation. **Valentina Greco:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Data curation, Conceptualization. **Salvatore Cuzzocrea:** Resources. **Rosalia Crupi:** Resources. **Enrico Gugliandolo:** Resources. **Alessandro Giuffrida:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Valentina Greco reports was provided by University of Catania. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466449.

## Data availability

Data will be made available on request.

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