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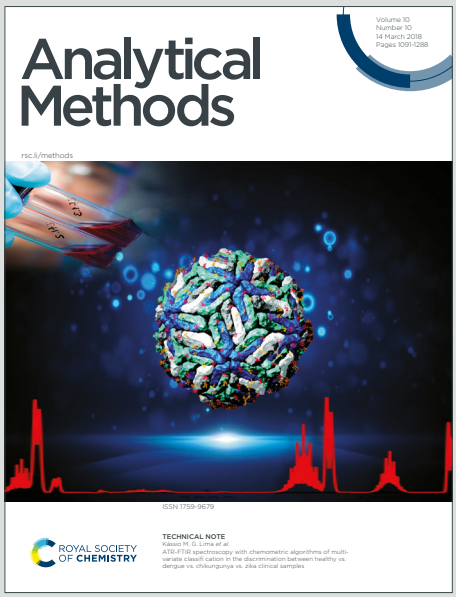




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# Fast and Sustainable Active Pharmaceutical Ingredient (API) Screening in Over-the-Counter and Prescription Drug Products by Surface-Assisted Plasma-Based Desorption/Ionization High-Resolution Mass Spectrometry

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## Abstract

Fast chemical analysis of pharmaceutical preparations is important for quality assurance, counterfeit drug detection, and consumer health. While quantitative methods such as high-performance liquid chromatography mass spectrometry (HPLC-MS) are powerful, there is a need for sustainable and environmentally friendly methods, which require less chemicals and produce less waste. Here, solvent-free ambient desorption/ionization (ADI) MS methods are attractive because they do not require time-consuming chromatography, produce little to no chemical waste, and, thus, contribute to green chemistry practice. In addition, sample throughput can be higher compared to HPLC-MS. In this study, a method for direct analysis of single- and multi-agent drugs using a plasma-based ADI source (flowing atmospheric-pressure afterglow, FAPA) coupled to high-resolution (HR) MS was developed and optimized for best performance. The approach is rapid and only requires analytes to be in solution (only a few µL) before application onto thin-layer chromatography (TLC) surfaces, specifically dimethyl (RP2-) and cyano (CN-) modified silica, for surface-assisted (SA) FAPA-HRMS measurements. No chromatographic separation was required, and the TLC plates served only as sample carriers. A broad variety of 19 active pharmaceutical ingredients

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(APIs) was carefully selected to cover analgesics, anesthetics, antibiotics, antiepileptics, calcium channel blockers, diuretics, expectorants, opioids, peripheral vasodilators, stimulants, and sympathomimetics. Fast screening and identification of APIs was performed by SA-FAPA-HRMS. Typically, the protonated molecular ion ($[M+H]^+$) was the most abundant species, while some compounds (codeine, metamizole, phenoxymethylpenicillin, and torasemide) did show some degree of fragmentation. As a proof-of-principle application, benzocaine was directly detected in saliva samples post-intake of a lozenge. Time-resolved semi-quantitative screening was performed. The limit of detection for benzocaine in saliva was 8 ng/mL (48.4 fmol) using internal standard calibration and CN-HPTLC plates. In addition, direct quantification of artificially spiked saliva was performed with minimal sample preparation. Here, SA-FAPA-HRMS with a CN-HPTLC sample substrate yielded best performance (20.02±0.52 µg/mL, RSD=2.6%, deviation of -1.9% from the theoretical value) compared to RP2-TLC (18.97±1.37 µg/mL, RSD=7.2%, -7.0%), and HPLC-UV (18.51±0.03 µg/mL, RSD=0.2%, -9.3%) results. In conclusion, SA-FAPA-HRMS is considered attractive for rapid and sustainable analysis of pharmaceuticals with potential in non-invasive patient monitoring.

Keywords: ambient desorption/ionization, mass spectrometry, pharmaceuticals, green chemistry

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## Introduction

Analytical sciences play an integral part to ensure the safety and effectiveness of pharmaceuticals. For example, pharmacopoeias strengthened purity testing, which has led to an increased use of high-performance liquid chromatography (HPLC) in quality control laboratories.[1] While HPLC systems with UV detectors were used early on, the invention of electrospray ionization (ESI)[2] lead to the possibility to interface HPLC and mass spectrometry (MS)[3], which, in turn, enabled structure identification, quantitation and impurity testing in complex samples. While powerful HPLC-MS methods for the characterization of different drug delivery systems exist, they share two limitations: *i)* the requirement to use substantial amounts of solvents for sample preparation, analyte extraction/matrix removal (if applicable) and liquid chromatography, and *ii)* they are very time-consuming because of the required steps mentioned before. Here, there is a need for faster, more sustainable, and environmentally friendly methods, which require less chemicals and produce less waste. A promising approach compared to classical chromatography-based method is ambient desorption/ionization mass spectrometry (ADI-MS). While a very popular ADI source is solvent-based (desorption electrospray ionization, DESI)[4] other methods operate solvent free and are laser- or plasma-based. One example of a plasma-based ADI source is DART (direct analysis in real time).[4, 5] DART-MS was used for identification of pharmaceuticals, detection of counterfeit drugs, and screening of drugs of abuse.[6-9] Further applications were recently reviewed.[10-13] In addition to DART, low-temperature plasma (LTP)-MS[14] was used, for example, for the direct detection of active ingredients in Coartem (artemether, lumefantrine) and Malarone (atovaquone, proguanil hydrochloride) antimalarial tablets.[15] A promising plasma-based alternative to DART and LTP is the flowing atmospheric-pressure afterglow (FAPA) probe coupled to MS, which was developed in the laboratories of G. M. Hieftje.[16-18] After its introduction, the original pin-to-plate FAPA source geometry was further refined into a pin-to-capillary design that improved overall performance in terms of lower mass spectral background, reduced analyte oxidation and improved sensitivity.[19] For

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improved sample introduction of solutions, vapor or aerosols, a so-called halo-FAPA source design was developed.[20]

One common approach in ADI-MS is to probe dried residues of liquid samples from solid support materials readily available in the laboratory (microscope slides, stainless steel plates/mesh, filter paper). In recent years, there is interest to use thin-layer chromatography (TLC) plates as sample support materials. For example, TLC plates were used with DART-MS,[21, 22] LTP-MS,[23] and DESI-MS[24-28] for the analysis of a wide range of substances after planar separation. This included purely qualitative approaches but also quantitative detection of organophosphorus insecticides[22] and semiquantitative imaging of biological extracts, respectively.[28] In addition, a so-called surface-assisted FAPA high-resolution MS (SA-FAPA-HRMS) method used TLC plates simply as sample substrates (without the need of a planar chromatography step) for the direct analysis of caffeine in beverages (energy drink, Coca-Cola, coffee, and black tea). Quantitation of caffeine in these beverages was performed by merely scanning the FAPA probe across the TLC plate and using co-deposited $^{13}C_3$-caffeine for internal calibration.[29] We have shown that a preceding planar chromatography step on a TLC plate can be beneficial in some FAPA-MS applications to remove matrix or potential interferences.[29, 30] Others have used a preceding planar chromatography step to remove the sample matrix and because high-mass resolution was not available. For example, Ceglowski *et al.* used a combination of planar chromatography, low-cost laser ablation and FAPA ionization coupled to a quadrupole ion trap MS to characterize pyrazole derivatives, nicotine, sparteine, and an extract from a drug tablet (paracetamol, propyphenazone, and caffeine).[31] In a series of studies, innovative techniques, including sheath-flow probe electrospray ionization (sfPESI) [32, 33] and paper spray ionization [34-37] demonstrated advancements in biofluid analysis and forensic applications using ambient mass spectrometry. Käser et al. reported advancements in online-breath analysis using secondary electrospray ionization-mass spectrometry (SESI-MS) for the detection of endogenous compounds in exhaled breath.[38] Additionally, dielectric barrier discharge ionization mass spectrometry enabled

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direct analysis of fentanyl analogs in blood and plasma samples [39]. Further applications of ADI-MS including analysis of drugs and toxins were recently reviewed by Henderson et al.[40].

In this work, we present a proof-of-principle study for the direct characterization of a broad variety of active pharmaceutical ingredients (APIs) in over-the-counter and prescription drugs using functionalized thin-layer surfaces (without the need of a planar chromatography step) and plasma-based desorption/ionization coupled to high-resolution mass spectrometry (HRMS). To also demonstrate direct and non-invasive drug monitoring possibilities, a semiquantitative and time-resolved screening for benzocaine was performed using saliva. Benzocaine was selected because it is freely available to consumers, often used during the cold season, and saliva sample collection is rapid and non-invasive. Sample preparation and the use of solvents and chemicals was kept at a minimum across all investigations. SA-FAPA-HRMS results are compared to and validated by HPLC.

## Materials and methods

**Reagents.** Methanol (HPLC grade) was purchased from Fisher Chemicals (Hampton, NH, USA). Benzocaine (98% chemical purity) was obtained from Acros Organics (Geel, Belgium) and $D_4$-benzocaine (chemical purity 98%, isotopic purity 99.9%) was obtained from Toronto Research Chemicals (Toronto, Canada). Other active pharmaceutical ingredients (APIs) were purchased from pharmacies. The most important information on the APIs is summarized in Table 1.

**Preparation of standard solutions.** The preparation of API standard solutions was kept to a minimum. Drug capsules were weighed and grinded before dissolving a small amount (1.4-10.4 mg) in methanol (1-10 mL depending on API content). After 15 minutes of ultrasonic treatment, solutions were microfiltered (0.45 µm) to eliminate insoluble filler materials. The concentrations related to the active ingredient content of each capsule are presented in Table 2. In addition, a standard solution series with analytically pure benzocaine was prepared, ranging from 10 ng/mL to 100 µg/mL. For HPLC validation experiments, two sets

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of benzocaine standards were prepared (see supporting information for details). A methanolic internal standard (IS) solution was prepared containing 1 µg/mL $D_4$-benzocaine.

**Preparation of saliva samples.** This study involved the collection of saliva samples from a healthy volunteer and was performed following the principles of the Helsinki Declaration. It was approved by the Ethics Committee of the University of Siegen, Germany, under reference number LS_ER_22_2025. First, blank saliva before API intake was collected using Salivette sample tubes (SARSTEDT AG & Co., Nümbrecht, Germany). To collect trapped saliva from the cotton sponge, the sample tube was centrifuged at a speed of 3200 rpm (revolutions per minute) for two minutes using an EBA 20 centrifuge (Andreas Hettlich GmbH & Co. KG, Tuttlingen, Germany). The saliva was stored at -18 °C until use to avoid changes in composition due to aging processes.

Samples for the benzocaine screening experiments were collected from a healthy volunteer immediately after consumption of a lozenge and at time intervals of 10 min for a total of 120 min after consumption (total of 13 samples). On days saliva samples were collected, only water and caffeine-free tea were consumed, and sampling was done only before large meals to minimize interferences.

**Table 1** Information on the APIs included in this study. Quantitative data in the tablets are based on information from the drug packaging.

| API | Drug name | Brand | $m\%_{API\ in\ capsule}$ [a] | $m_{weighed}$ [mg] | $V_{MeOH}$ [mL] | $C_{API}$ [µg/mL] |
|---|---|---|---|---|---|---|
| Acetaminophen | Oxycodone-APAP | CVS/Pharmacy | 61.4 | 8.1 | 3.0 | 1658 |
| | Migraene-Kranit® | Krewel Meuselbach | 33.2 | 9.1 | 1.0 | 3021 |
| Ambroxol | Mucusolvan® | Sanofi | 34.6 | 1.8 | 2.0 | 311.4 |
| Amlodipine | Amlodipin | Dexcel® Pharma | 2.5 | 5.3 | 2.0 | 66.2 |
| Aspirin | Aspirin® Complex | Bayer | 18.2 | 7.0 | 10 | 127.4 |
| | Dolviran® | Bayer | 31.5 | 10.4 | 1.0 | 3276 |
| Benzocaine [b] | Dolo-Dobendan® | Reckitt Benckiser | 0.4 | - | - | - |
| Caffeine | Migraene-Kranit® | Krewel Meuselbach | 14.1 | 9.1 | 1.0 | 1283 |
| | Dolviran® | Bayer | 7.9 | 10.4 | 1.0 | 821.6 |
| Codeine | Dolviran® | Bayer | 1.5 | 10.4 | 1.0 | 156.0 |
| Diclofenac | Diclo KD® 75 akut | DR. KADE | 31.4 | 3.8 | 1.0 | 1193 |
| Doxycycline | Doxycyclin 100 | 1A Pharma | 51.4 | 1.8 | 2.0 | 462.6 |
| Ethaverine | Migraene-Kranit® | Krewel Meuselbach | 3.3 | 9.1 | 1.0 | 300.3 |
| Ibuprofen | IBU 600 | 1A Pharma | 84.0 | 2.1 | 2.0 | 882.0 |
| Metamizol | Novaminsulfon | Zentiva | 76.5 | 2.8 | 2.0 | 1071 |

| API | Drug name | Brand | $m\%_{API\ in\ capsule}$ [a] | $m_{weighed}$ [mg] | $V_{MeOH}$ [mL] | $C_{API}$ [µg/mL] |
|---|---|---|---|---|---|---|
| Naproxene | Naproxene | CVS/Pharmacy | 95.5 | 2.7 | 3.0 | 859.5 |
| Oxycodone | Oxycodone-APAP | CVS/Pharmacy | 0.9 | 8.1 | 3.0 | 24.3 |
| Phenacetin | Dolviran® | Bayer | 31.5 | 10.4 | 1.0 | 3276 |
| Phenobarbital | Migraene-Kranit® | Krewel Meuselbach | 5.0 | 9.1 | 1.0 | 455.0 |
| | Dolviran® | Bayer | 3.9 | 10.4 | 1.0 | 405.6 |
| Penicillin V | Pen 1,5 Mega | 1A Pharma | 86.5 | 1.4 | 2.0 | 605.5 |
| Propyphenazone | Migraene-Kranit® | Krewel Meuselbach | 24.9 | 9.1 | 1.0 | 2266 |
| Pseudoephedrin | Aspirin® Complex | Bayer | 1.1 | 7.0 | 10 | 7.7 |
| Torasemide | Torasemid | 1A Pharma | 6.2 | 6.5 | 2.0 | 201.5 |

[a] Mass percent based on information from the drug packaging;[b] Consumed as Dolo-Dobendan® (Reckitt Benckiser) as a whole lozenge and probed from saliva samples

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**HPTLC preparation.** Glass-backed TLC plates (3.3 cm x 3.3 cm, cut from 10 cm x 20 cm standard plates, CN-HPTLC and RP2-TLC, both MACHEREY-NAGEL, Düren, Germany) were used as sample carriers. A Linomat V spray-on application system (CAMAG, Muttenz, Switzerland) was used for sample deposition at a dosing speed of 50 nL/s. The spraying distance was set to 2.4 mm between syringe tip and sample carrier surface. The step size (distance travelled between application of different sample spots) was kept constant at 5 mm. Samples were analyzed in triplicate, i.e., three 1 µL samples were sprayed onto the plates in proximity. It is important to note that no time-consuming drying step was required and no chromatographic step was performed.

**Instrumentation.** For mass spectrometric analysis, an Exactive HCD Orbitrap (Thermo Fisher Scientific, Bremen, Germany) was coupled to a home-built pin-to-capillary FAPA ionization source. The mass spectrometer was calibrated weekly with a conventional ESI source. For positive ion mode calibration, the Pierce® LTQ ESI positive ion calibration solution (Thermo Fisher Scientific, Bremen, Germany) with caffeine (20 µg/mL), Met-Arg-Phe-Ala (MRFA, 1 µg/mL) and Ultramark 1621 (0.001%) in an aqueous solution of acetonitrile (50%), methanol (25%) and acetic acid (1%) was used. For negative ion mode calibration, the Pierce® LTQ ESI negative ion calibration solution (Thermo Fisher Scientific, Bremen, Germany) was used. Instead of caffeine and MRFA, the solution contained sodium dodecyl sulfate (2.9 µg/mL) and sodium taurocholate (5.4 µg/mL). The mass resolution was set to 50,000 (at 200 *m/z*). The day-to-day mass measurement uncertainty did not exceed 2 ppm in the positive ion mode and 4 ppm in the negative ion mode, respectively. To ensure high mass accuracy, positive ion mode measurements included lock masses of ubiquitous species (phthalic anhydride fragment at 149.2033 *m/z*; n-butylbenzenesulfonamide at 214.0896 *m/z*). In negative ion mode, no comparable species were detectable at sufficient abundance.

The FAPA ionization source discharge chamber consisted of Macor® ceramic (Schröder Spezialglas, Ellerau, Germany). A stainless-steel pin cathode (1.6 mm outer diameter (o.d.),

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100 mm length, sharpened tip) as well as a capillary-anode (1.6 mm o.d., 1.0 mm inner diameter (i.d.), 15 mm length) were screwed into the discharge chamber. The cathode-to-anode distance was 7.5 mm. To generate the helium discharge, a negative potential was applied to the cathode through a 5 kΩ resistor, and a DC power supply (Kepco, Flushing, NY, USA) was operated in current-controlled mode at a helium flow rate of 750 mL/min using grade 5 helium (Messer Industriegase GmbH, Siegen, Germany). At 25 mA, the resulting potential was around 650 V. For mounting the FAPA source on the MS, a bracket with a suitable connection that matches the conventional ESI source bracket on the instrument inlet was used. A motorized stage (Newport Corporation, Irvine, CA, USA) as part of the mounting device positioned the sampling surfaces underneath a curved ion transfer capillary (0.6 mm inner diameter, 40 mm extension). The stage was controlled with a custom LabVIEW (Version 11.0, 2011, National Instruments, Austin, TX, USA) program. For mass spectrometric experiments, an angle of 70° and a distance of 1.0 mm between the FAPA outlet capillary and the respective desorption surface was set. The distance between surface and ion transfer capillary was adjusted to 0.5 mm and the distance between ion transfer capillary and FAPA outlet capillary was kept at 1.0 mm. TLC plates were fixed on the motorized stage by a spring-loaded clamp.

**Molecular mapping and data analysis.** Two-dimensional (2D) molecular maps were generated by linear scanning of the TLC surface with a scanning speed of 0.3 mm/s in the x-direction and a line spacing of 0.5 mm in the y-direction. The LabVIEW program that was used to control the stage motion was also used to trigger the data acquisition for automated MS imaging. Individual x-line scans were summed to form 2-dimensional molecular maps by converting the time for one line scan to a distance based on a scan rate of 0.3 mm/s. The y-axis distance was determined by the total number of lines at 0.5 mm intervals. These parameters, combined with XICs abundances, were used to generate contour plot diagrams. In the contour plots, so-called regions-of-interest (ROI) were used and integrated, to gain abundance information for the individual APIs.

Exactive Tune software (Version 1.1 SP6, Thermo Scientific, Bremen, Germany) was used to control the MS and to acquire data. Analyte-selective information were acquired by extracted ion chronograms (XICs) within a *m/z*-range of ±4 ppm in the positive ion mode according to Table S1 and within a m/z-range of ±6 ppm in the negative ion mode according to Table S2. The differences were chosen based on the instrumental variations in the two ion modes. Further data processing was performed with MZmine 2.53 and Origin 2017 (OriginLab Corporation, Northampton, MA, USA).

**Safety considerations.** The experimental setup used for the experiments involved potentially harmful voltages and currents to operate the FAPA source. Connections between the power supply and the ionization source were insulated to prevent potential electric shocks. An acrylic enclosure was used to cover the FAPA source to prevent vaporized solvents and potentially toxic or corrosive chemicals and by-products from entering the laboratory atmosphere and to cover the experimental setup from contaminations and interferences.

## Results and Discussion

**Direct analysis of single-agent drugs on cyano and dimethyl surfaces using FAPA-MS.** Drug preparations can be differentiated into two groups depending on the number of active ingredients combined in one tablet. Monopreparations are pharmaceutical compositions that contain a matrix and only one active ingredient. When two or more active ingredients are combined (e.g., in a tablet) this approach is called combination preparation or combination drug. Fast qualitative screening of pharmaceutical preparations with a straightforward MS procedure would be desirable not only for quality assurance. In the first part of the study, the feasibility of direct and fast monopreparation analysis using SA-FAPA-MS was studied (monopreparations will be referred to as single-agent drugs throughout the text). For direct analysis, single-agent drugs were simply applied to carefully selected TLC surfaces as sample substrates. These sample substrates have advantages

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over conventional substrates such as glass or metal as was reported earlier.[29, 41] For example, improved reproducibility and reduced analyte diffusion were reported, which are critical for accurate quantitative results. The selection of the surface modification and solvent (methanol) in this study was based on previously published data demonstrating the excellent properties of CN- and RP2-modified surfaces for direct desorption/ionization mass spectrometry of small molecules.[41]

Figure 1A shows a direct comparison of the ion abundance of nine different APIs (see Table S1 for information on specific *m/z*) when probed with FAPA-MS directly from CN-HPTLC (green) and RP2-TLC (yellow) surfaces. Please note that a preceding planar chromatography step was not used. As a general observation it was found that the use of RP2-TLC for the APIs investigated in this study was beneficial over CN-HPTLC plates because the signals were mostly comparable or higher using RP2-TLC. The largest signal increase (from CN-HPTLC to RP2-TLC) was recorded for ambroxol ($[M+H]^+$, $m/z_{theo}$ 378.9838 for $^{79}Br^{81}Br$-species, positive ion mode). This observation is consistent with a previous study, in which the use of RP2 surfaces was found beneficial as well but for a different set of compounds (caffeine, acetaminophen, progesterone, and nicotine).[41] In Figures 1B and 1C, the APIs are ordered by the absolute amount of active ingredient applied onto the surface in nmol. For almost all compounds, a correlation between the applied amount on the surface and the resulting ion abundance after FAPA-MS analysis can be observed. Typically, in positive ion mode the protonated species ($[M+H]^+$) of the APIs were the most abundant species compared to, e.g., fragment ions. A slightly higher degree of fragmentation or adduct formation was observed for amlodipine, codeine, ibuprofen, metamizole, and phenoxymethylpenicillin. Specifically, the protonated species were not the predominant ions in the mass spectra (see Table S1 for detailed information). Among the studied compounds, doxycycline ($[M+H]^+$, $m/z_{theo}$ 444.1527, positive ion mode) was found to be an exception. This compound exhibited the lowest ion abundance in FAPA-MS for both TLC surfaces even though three other compounds including amlodipine ($[M-C_3H_8NO]^+$,

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*m/z*$_{theo}$ 334.0841 for $^{35}$Cl-species, positive ion mode) were probed at a lower nominal concentration (1 nmol of applied doxycycline vs. 0.117 nmol of applied amlodipine). Given the different structures and properties of the compounds mentioned above, a difference in ion abundance would not be surprising. Cleary, such comparisons should be treated with caution because the recorded ion signals are the result of a combination of factors (desorption/ionization efficiency, degree of fragmentation, ion transmission etc.). To further investigate the desorption and ionization behavior of doxycycline, comparative experiments with tetracycline were performed. This pair was selected because doxycycline and tetracycline are structural isomers that differ only in one substitution site of a hydroxyl group (-3,5,10,12,12a-pentahydroxy- vs. -3,6,10,12,12a-pentahydroxy-). While the signal of doxycycline was already small compared to other APIs, tetracycline was not detectable at all in positive ion mode (data not shown). A similar trend was found in negative ion mode (see Fig. 4 in section “comparison of positive and negative ion mode” for selected APIs.) While it is not the aim of the present study to investigate the influence of molecular differences on the desorption and/or ionization efficiency in ADI-MS in detail, this example illustrates possible effects of small molecular differences. Clearly, quantitative FAPA-MS methods will benefit from internal and matrix-matched standardization.

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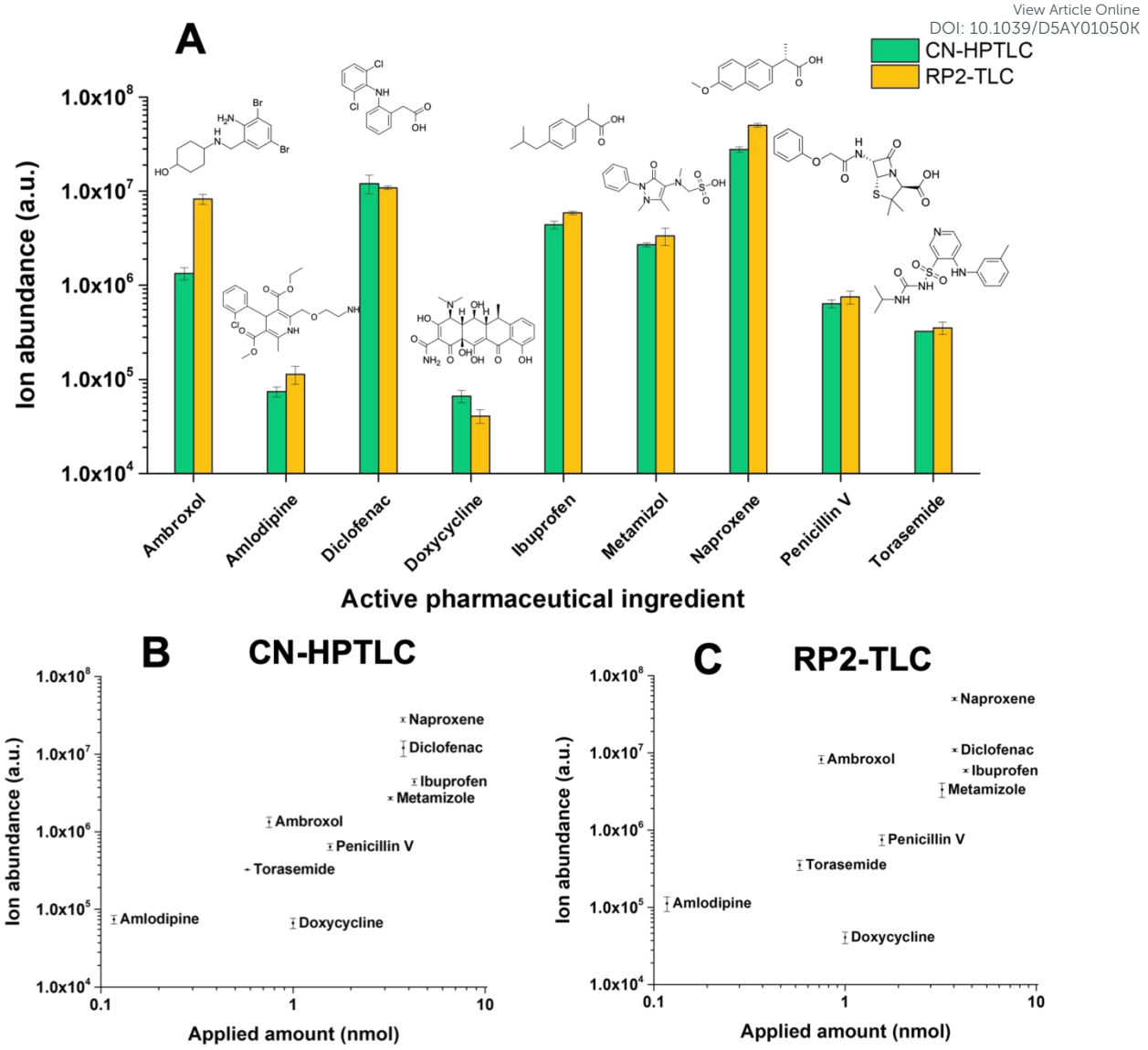


**Fig. 1** Direct API screening of single-agent drugs in tablets using SA-FAPA-HRMS and minimal sample preparation: Influence of sample substrate on ion abundance of nine APIs probed from CN-HPTLC (**A, in green**) and RP2-TLC (**A, in yellow**) surfaces without a preceding planar chromatography step. Overview of ion abundance vs. applied API amount (depending on the commercialized drug content dissolved in MeOH) after analyte desorption/ionization from CN-HPTLC (**B**) and RP2-TLC (**C**). Error bars correspond to n=3 replicates and represent the standard deviation. Note the logarithmic scaling. Signals are integrated molecular contour plots of analyte species according to Table S1.

**Analysis of multi-agent drugs on cyano and dimethyl surfaces.** Multi-agent or combination drugs contain two or more active ingredients and are often prescribed. A significant advantage is the reduced pill burden of patients making it easier to manage medication intake and thus reduce adverse side effects.[42] In the second part of the study, several multi-agent drugs, namely Aspirin® Complex, Dolviran®, Migraene-Kranit®, and

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Oxycodone-APAP (Table 1) were probed by SA-FAPA-HRMS. The inclusion of multi-agent drugs provides an opportunity to investigate the competitive behavior of different compounds with each other in terms of desorption and ionization efficiency. This so-called competitive ionization can be a challenge in ADI-MS and was reported, for example, for multi-analyte standards in a previous FAPA-MS study. [41] It was shown that the presence of high nicotine concentrations lead to significant signal losses for other simultaneously present analytes (e.g., progesterone).

For this study, two multi-agent drugs were probed that contain two APIs (and matrix), namely Aspirin® Complex and Oxycodone-APAP. SA-FAPA-HRMS results are summarized in Figures 2 and S1.

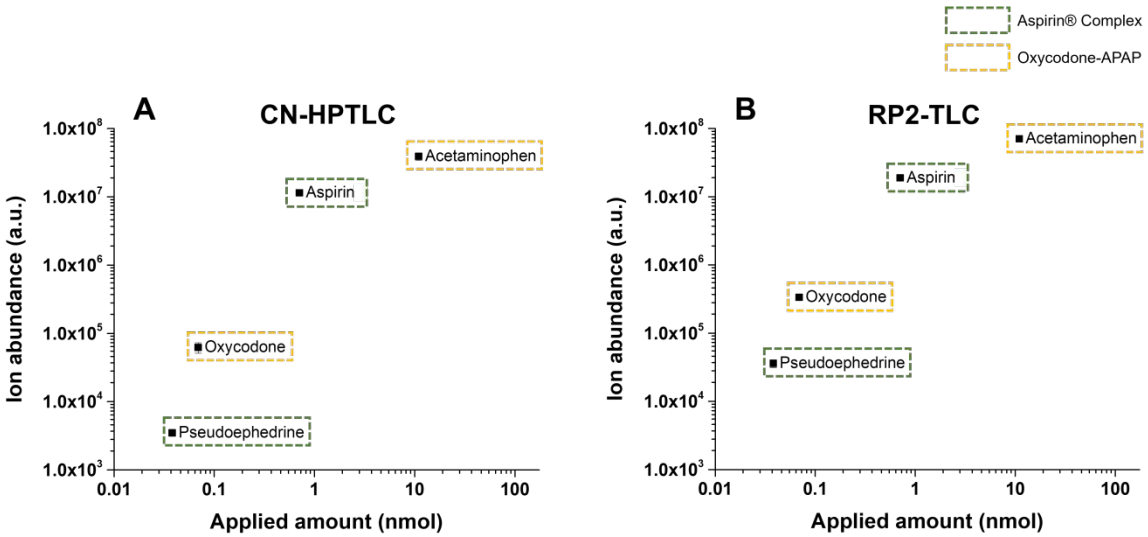


**Fig. 2** Direct API screening of multi-agent drugs in tablets using SA-FAPA-HRMS and minimal sample preparation: Overview of ion abundance vs. applied API amount (depending on the commercialized drug content dissolved in MeOH) after analyte desorption/ionization from CN-HPTLC (**A**) and RP2 TLC (**B**). Highlighted in green are the APIs in Aspirin® Complex (Aspirin and pseudoephedrine), highlighted in yellow are the APIs in Oxycodone-APAP (Oxycodone and acetaminophen). Error bars correspond to n=3 replicates and represent the standard deviation. Note the logarithmic scaling. Signals are integrated molecular contour plots of analyte species according to Table S1.

In the multi-agent drugs, both aspirin (0.707 nmol) and acetaminophen (10.96 nmol) are present in significantly larger amounts compared to pseudoephedrine (0.038 nmol) and oxycodone (0.069 nmol), respectively. When comparing the applied amounts of aspirin and

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pseudoephedrine to their corresponding mass spectral signals (see Figure 2), it can be concluded that the use of RP2 surfaces is favorable over CN-HPTLC. While significantly more aspirin, referred to as excess factor throughout the text (~20-fold more deposited aspirin compared to pseudoephedrine,), was applied to both TLC surfaces, an approximately 3300-fold higher signal for aspirin compared to pseudoephedrine was recorded when the FAPA source probed the CN surface. In contrast, the aspirin signal was approximately 520-fold higher using RP2-TLC plates as sample substrate. A consistent trend is observed for oxycodone APAP. Here, the excess factor is approximately 159 for administered acetaminophen compared to oxycodone. The signal on the CN surface is 630-fold higher for acetaminophen compared to oxycodone. On the RP2 surface, a 210-fold higher signal was measured for acetaminophen, which is quite close to the respective excess factor. In general, stronger analyte signals were obtained using RP2 surfaces compared to CN-HPTLC plates, which is consistent with our previous results.[41]

After the multi-agent drug screening with two APIs, more complex multi-agent drugs with five APIs, namely Dolviran® and Migraene-Kranit®, were studied with FAPA-MS. Information on the compounds and detected ions are given in Tables 1 and S1. In Figure 3, analyte ion responses of APIs in Dolviran® and Migraene-Kranit® are summarized for two different surfaces. Contrary to single-agent drugs (Fig. 1), a mixture of compounds was presented to the desorption/ionization source. Here, matrix effects due to e.g., competitive ionization or preferential desorption effects could potentially influence the results. These effects typically start to manifest with increasing analyte concentration and/or matrix and fundamental parameters such as proton affinity (PA) and enthalpy of vaporization ($\Delta H_{vap}$) are important to consider.[41] When comparing the two molecules present at highest amounts in Dolviran®, namely aspirin (18.18 nmol applied) and phenacetin (18.28 nmol applied), ion abundance differences are readily recognizable (Fig. 3A,B). The phenacetin signal is approximately 8-fold higher with CN-HPTLC and 19-fold higher with RP2-TLC compared to aspirin even though the nominal concentrations in the drug formulation are very similar. This signal

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difference is assumed to be a result of the different physicochemical properties of the molecules. Phenacetin is a 4-substituted acetanilide derivative and shows structural similarities to acetaminophen (difference is the 4-ethoxy group compared to the 4-hydroxy group in acetaminophen).

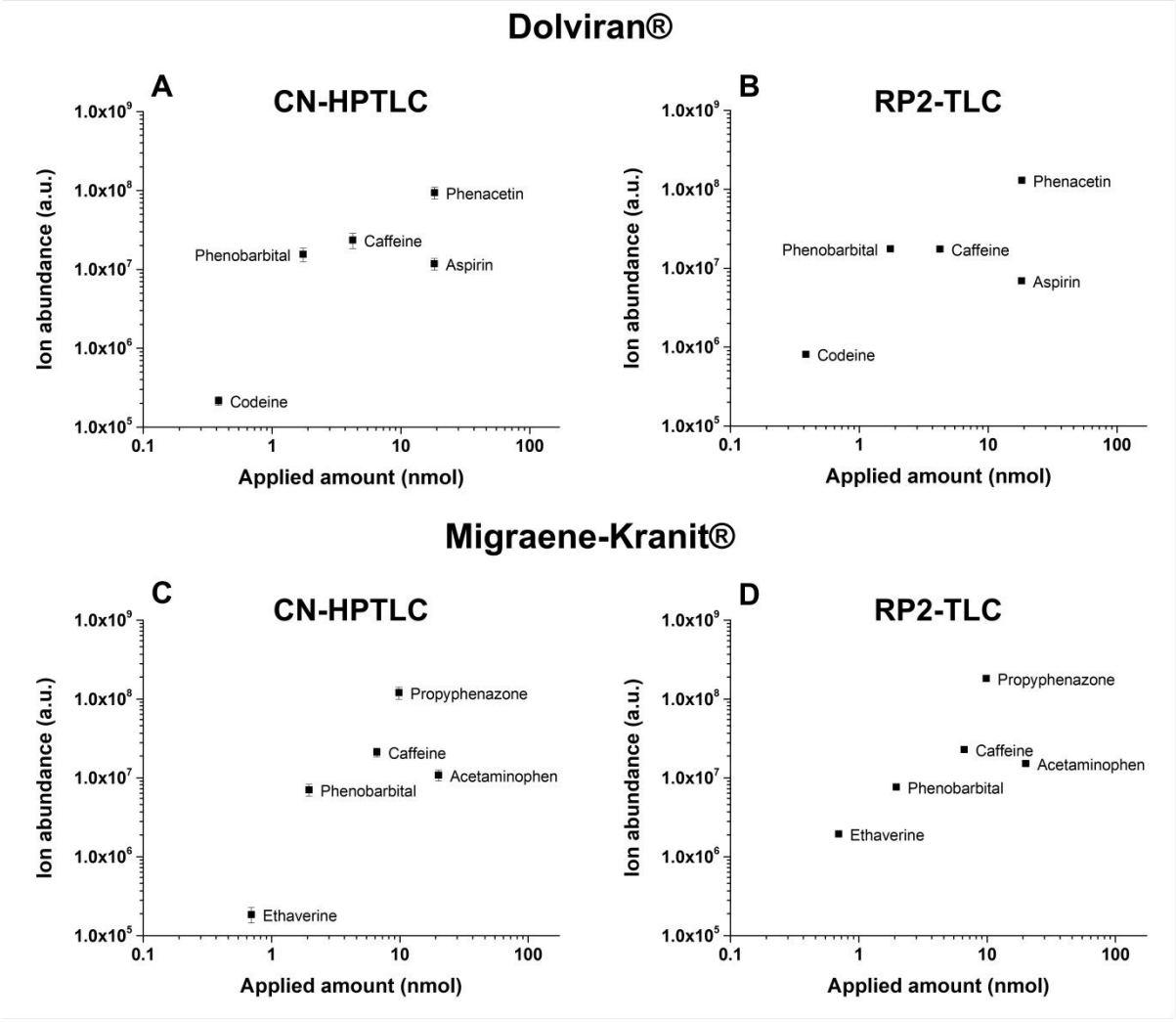


**Fig. 3** Direct API screening of multi-agent drugs in tablets using SA-FAPA-HRMS and minimal sample preparation: Overview of ion abundance vs. applied API amount (depending on the commercialized drug content dissolved in MeOH) for Dolviran® (upper section) after analyte desorption/ionization from CN-HPTLC (**A**) and RP2-TLC (**B**) and for Migraene-Kranit (lower section) after analyte desorption/ionization from CN-HPTLC (**C**) and RP2-TLC (**D**). Error bars correspond to n=3 replicates and represent the standard deviation. Note the logarithmic scaling. Signals are integrated molecular contour plots of analyte species according to Table S1.

Because no reliable PA values for phenacetin were found in the literature, acetaminophen was used to estimate the PA value (because the protonation sites stay almost the same).

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The PA values for the most favored protonation sites in the acetaminophen range from 887 to 915 kJ/mol.[43] For aspirin, the PA for the most preferred site of protonation, precisely the carbonyl oxygen of the carboxylic acid group, was obtained by density functional theory (DFT) calculations with a value of 867.8 kJ/mol.[44] If one assumes that the PAs of acetaminophen can serve as an approximation for phenacetin, the latter should have higher PA compared to aspirin. This, in turn, would result in a preferential proton transfer ionization of phenacetin in the presence of aspirin. In fact, mass spectra of the multi-agent drug did reveal that the most abundant ion species of phenacetin was in fact the protonated $[M+H]^+$ molecular ion. Aspirin on the other hand did undergo deacetylation and was mainly detected as the $[M\text{-}C_2H_3O_2]^+$-species. The deacetylation may also be induced by prior protonation, but this protonation site was not mentioned in the DFT simulations.

Another API in Dolviran® is caffeine (4.23 nmol applied, 3rd highest nominal concentration in the drug formulation), which was detected as the protonated molecular ion ($[M+H]^+$) and little fragmentation. Compared to caffeine, the amount of phenacetin applied to the surface was 4.3 times higher. This corresponds approximately to the measured difference in ion abundance, which was about 4.0 times higher for phenacetin compared to caffeine. All the other APIs in the drug were successfully detected as well.

In the second multi-agent drug Migraene-Kranit®, acetaminophen (19.99 nmol applied) and propyphenazone (9.84 nmol applied) were the APIs with the highest nominal concentration. Despite the fact that the applied amount of acetaminophen is more than twice as high as the amount of propyphenazone, acetaminophen exhibited a significantly lower ion abundance and with both surfaces (Fig. 3C, 3D: 11-fold lower with CN and 12-fold lower with RP2 plates). For both molecules the $[M+H]^+$-species was the most abundant ion in the mass spectrum. Although the PAs for acetaminophen are available for its favored protonation sites (887 to 915 kJ/mol[43]) we could not find literature data for PA of propyphenazone itself. The basic structure of propyphenazone is a heteroaromatic pyrazole, or more precisely the pyrazolone. The CRC Handbook of Chemistry and Physics gives an overview of the PAs of

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various pyrazole derivatives.[45] Comparing the different derivatives, it is evident that the PA increases with increasing alkyl or phenyl substitution (Table S3). For instance, the dialkyl and diphenyl derivatives of pyrazole show significantly higher PAs (927.3 – 946.3 kJ/mol) than the unsubstituted pyrazole (894.1 kJ/mol).[45] It was therefore assumed here, that propyphenazone would have a higher PA compared to acetaminophen. This, in turn, would lead to a more efficient proton transfer ionization of propyphenazone compared to acetaminophen, which is in agreement with the ion abundances of the protonated molecular ions in this work.

In terms of the two different TLC surfaces, only minor differences in the ion abundances were observed. Overall, RP2-TLC plates did show slightly better performance for the APIs at higher concentration but really helped to detect compounds at lower amounts (except for phenobarbital). For ethaverine, codeine, pseudoephedrine and oxycodone, the ion abundances were found to differ significantly depending on the surface used. Sensitivity for these compounds was better when they were applied onto and desorbed from RP2-functionalized surfaces. This finding is in good agreement with previous results for acetaminophen and progesterone on RP2-TLC.[41]

**Comparison of positive and negative ion mode.** Previous studies on surface-assisted FAPA-MS focused mainly on positive ion mode detection. The molecular variety and different functionalities of the APIs included in this study, however, gave the opportunity to compare the performance of the method in both positive and negative ion mode. For this comparative study, APIs were selected that are either present as alkali salts or that feature a carboxyl group. Figure 4 compares the FAPA-HRMS results in positive and negative ion mode for both TLC types. Some differences are evident, though less clearly pronounced than initially expected. For metamizole and naproxene, the detection in positive ion mode did yield significantly higher analyte signals independent of the desorption surface. Diclofenac detection in positive ion mode was beneficial with a higher signal when the CN-HPTLC sample substrate was used. Penicillin V detection in negative ion mode after deposition on

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CN-HPTLC surfaces did show a better performance compared RP2-TLC.In contrast, the Penicillin V signal was higher in positive ion mode when the RP2 surface is used. While a detailed mechanistic study is beyond the scope of this study, this would be an interesting topic to follow up on. In future applications, both detection modes should be carefully evaluated during method optimization for best performance.

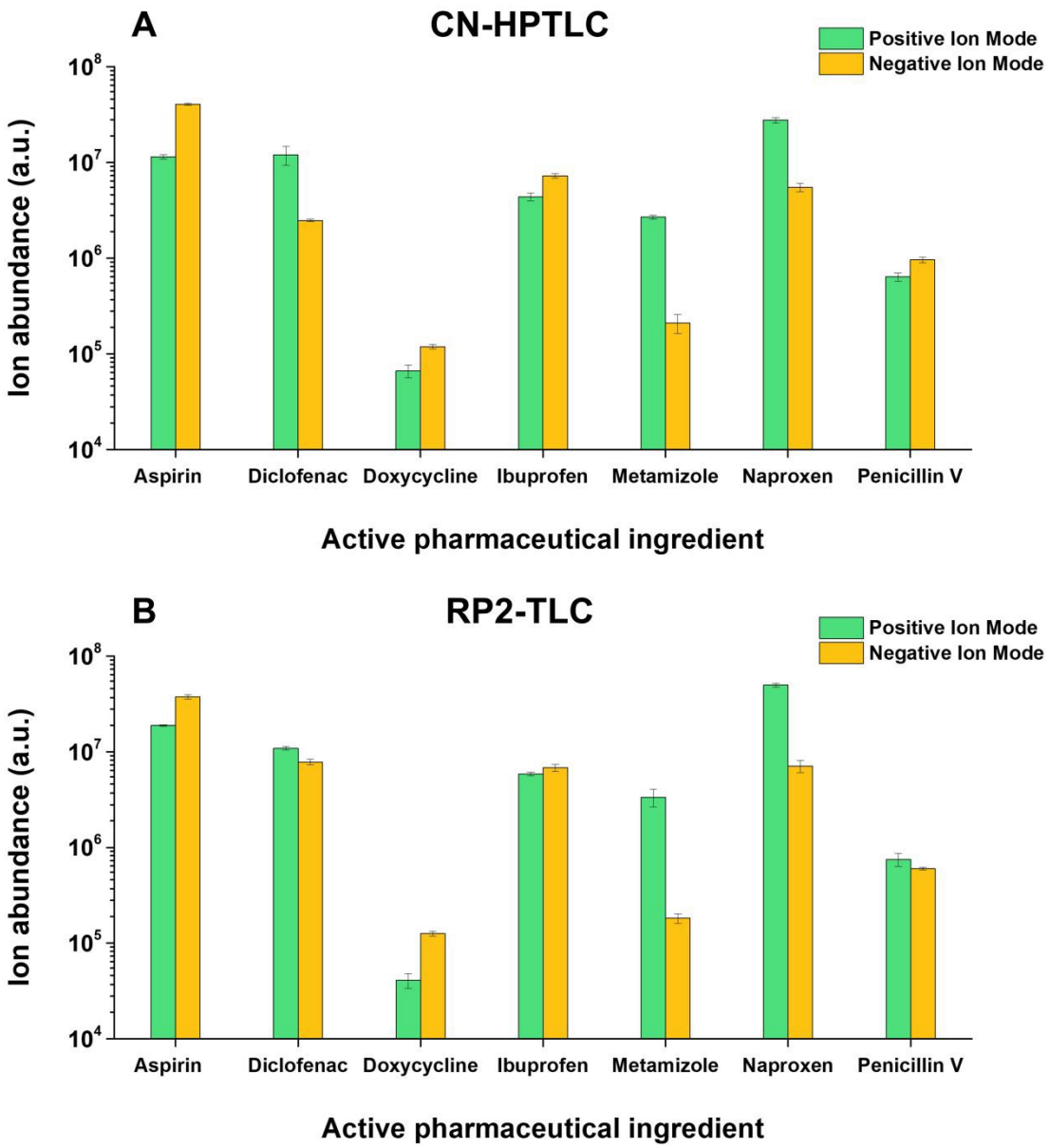


**Fig. 4** Ion abundances of selected APIs in positive (green) and negative (yellow) ion mode based on desorption and ionization from CN-HPTLC (A) and RP2-TLC (B). Error bars correspond to n=3 replicates and represent the standard deviation. Note the logarithmic scaling.

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**Direct (semi-)quantitative benzocaine screening from saliva samples.** The use of drugs necessitates diagnostic analysis, especially in clinical trials and for patient monitoring and safety. This usually includes bio fluids such as whole blood, serum, plasma, and urine but also sweat or saliva. The latter both offer the possibility of non-invasive sampling, which is gentle on patients and particularly attractive for pediatrics. So-called salivettes have become established for collecting saliva samples. Here, a cotton sponge is used to absorb salivary fluid, which is then collected by centrifugation. In terms of a proof-of-principle study, SA-FAPA-HRMS was tested for time-resolved saliva screening to semiquantitatively monitor salivary benzocaine. Saliva samples were taken over a defined period after consumption of the lozenge containing the active ingredient which is available over the counter. The samples were analyzed directly in 10-minute increments without further preparation. Figure 5 illustrates the workflow and representative results over a 70-minute period.

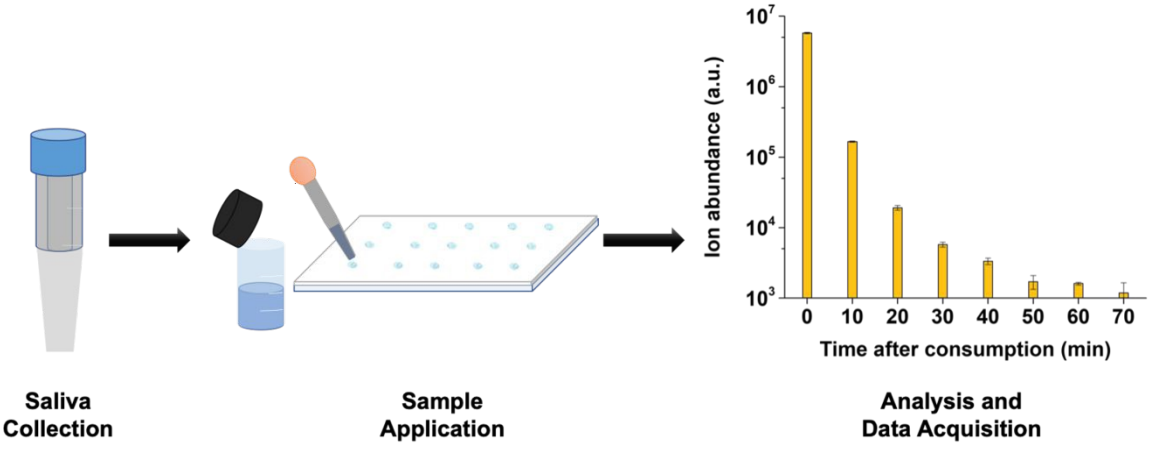


**Fig. 5** Workflow of the non-invasive, semiquantitative benzocaine screening in saliva samples. The samples are collected via salivettes and centrifuged. After application of 1 µL of untreated saliva per spot (n=3 per time increment) the RP2 surface is analyzed by SA-FAPA-MS and the data are further evaluated. Error bars correspond to n=3 replicates and represent the standard deviation. Note the logarithmic scaling in the right graph.

The benzocaine signal decays and reaches its minimum after 70 min which corresponds to a number of eight saliva samples that are included. In the five remaining samples (80 – 120 min after consumption), no benzocaine was detectable anymore.

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For better interpretation of these semiquantitative data, a look at the performance parameters of the corresponding SA-FAPA-HRMS method is important. Table 3 compares the respective parameters using RP2-TLC or CN-HPTLC as the sample substrate either with or without the use of an IS. The use of CN-modified surfaces achieved slightly better limits of detection (LODs) and limits of quantification (LOQs), as does the use of an IS. Hence, the best performance regarding sensitivity was obtained by internal standardization and desorption from CN-HPTLC surfaces with an LOD of 8 ng/mL. The best achievable LOD based on RP2-TLC was 11 ng/mL. With respect to the results shown in Fig. 5 based on desorption from RP2-TLC without using an IS, the lowest salivary benzocaine concentration corresponding to 70 min after lozenge consumption can be estimated to be approximately higher than 13 ng/mL. For comparison of the semi-quantitative findings with quantitative results, the saliva samples were also analyzed by HPLC-MS using internal standard calibration (Supporting Information). Here, benzocaine in the first saliva sample (0 min) was very high (approximately 36.5 µg/mL) and out of the calibration range (10 ng/mL – 100 ng/mL) of the method. Nevertheless, the rapid benzocaine decrease in saliva could be monitored all the way to falling below the LOD after 60 min post-intake. The HPLC-MS methods $R^2$ was 0.9973 and the achieved LOD was 6.1 ng/mL. Starting at 60 min post-intake and later in time, signals were below the LOQ and LOD determined for the HPLC-MS method. As the method does not offer any separation options, interference cannot be ruled out. These can be caused by the body's own molecules or metabolic products as well as by isobaric environmental substances. In multi-drug agents, different active substances can also be degraded into the same metabolites, which can also lead to interferences and misinterpretations during detection and data analysis. Especially with regard to clinical applications, this must be taken into account for accurate data interpretation.

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**Table 2** Performance parameters of the SA-FAPA-MS methods for Benzocaine analysis using either RP2-TLC or CN-HPTLC as the sample carrier surface with or without including an internal standard.

| Sample Carrier Surface | $R^2$ | LOD [ng/mL] | LOQ [ng/mL] |
|---|---|---|---|
| **No internal standard used [a]** | | | |
| RP2-TLC | 0.9914 | 13 | 42 |
| CN-HPTLC | 0.9965 | 12 | 40 |
| **Sample Carrier Surface** | **$R^2$** | **LOD [ng/mL]** | **LOQ [ng/mL]** |
| **Internal standard used [b]** | | | |
| RP2-TLC | 0.9996 | 11 | 38 |
| CN-HPTLC | 0.9978 | 8 | 28 |

[a] Performance parameters based on calibration using 10 ng/mL – 100 µg/mL benzocaine standards; [b] Performance parameters based on calibration using 10 ng/mL – 100 µg/mL standards, each spiked with a $D_4$-Benzocaine standard (1 µg/mL)

For direct accurate benzocaine quantification in saliva samples with SA-FAPA-MS, an artificial saliva sample with a known benzocaine amount (20.4 µg/mL) was prepared and analyzed using an IS calibration approach including four standards (Supporting Information). Based on CN-HPTLC as the sampling substrate, a calibration curve with an $R^2$ of 0.9922 was obtained. Further calculation of the benzocaine content in the salivary sample resulted in a concentration of 20.02 ± 0.52 µg/mL. This correlates to a small deviation of -1.9% from the theoretical value of 20.4 µg/mL. Additionally, the same procedure was performed using RP2-TLC as sampling substrate. Here, an $R^2$ of 0.9949 was achieved and the calculated concentration was 18.97 ± 1.37 µg/mL, which correlates to a deviation of -7.0% from the theoretical value. For validation purposes, also a HPLC-UV method was used (Supporting Information) on the same artificially prepared saliva sample using external standard calibration. Here, a benzocaine concentration of 18.51 ± 0.03 µg/mL was determined

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(deviation of -9.3% from theoretical value). When comparing the SA-FAPA-MS results with the HPLC-UV validation results, deviations of +8.2% for CN-HPTLC based analysis and +2.5% for RP2-TLC based analysis were achieved.

It has been shown above that both the semiquantitative and the accurate quantitative approach can yield results for the direct analysis of benzocaine in untreated saliva samples that are in good agreement with standard HPLC methods. We believe that these promising results make FAPA-HRMS an attractive tool for fast API screening, especially due to the non-invasive nature of collecting patient samples when using saliva. Particularly orally applied drugs are suitable for this method, but also other biofluids would be accessible for this purpose, which can significantly increase the spectrum for possible analytes.

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## Conclusion

SA-FAPA-HR-MS has been successfully applied to the direct analysis of pharmaceutical products. As a solvent-free and rapid screening method, it can be a promising addition to the toolbox for pharmaceutical analysis. API detection was successfully demonstrated for single-agent drugs and multi-agent drugs. This is particularly advantageous for the latter because extraction or separation steps were not required, and all APIs could be detected side by side (provided that no isomers are to be investigated). Furthermore, minimum amounts of liquid sample solution (1 µL) were required. Simultaneous API detection in multi-agent drugs, even with significant concentration differences within a tablet, was feasible using surface-assisted desorption/ionization. This method proves effective over a broad dynamic range and efficiently detects a variety of drug molecules, with the choice of negative or positive ion mode having little impact on results, rendering it a practical and reliable routine approach.

Sensitivity was demonstrated using benzocaine detectable at low ng/ml (ppb), corresponding to low fmol application, and highlighting the suitability of the method for diagnostics. It was successfully employed to track benzocaine levels in saliva after oral administration, enabling semi-quantitative monitoring of its decrease over a defined time frame. Calibration-based quantification in an artificial sample showed promising diagnostic potential with minimal, non-invasive efforts, including validation against established methods like HPLC-UV.

SA-FAPA-MS holds promise as a rapid mass spectrometry technique for drug analysis and direct diagnostics. Its potential extends beyond saliva to other biofluids, and it may find applications in kinetic studies for reaction control in the future.

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## Data availability

Supporting data are partially included in the Supplementary Information. Additional data that support the findings of the saliva study are available from the corresponding author upon reasonable request. Participants of this study did not give written consent for their data to be shared publicly.

## Compliance with ethical standards

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The reference number of the Institutional Ethics Board for this study is LS_ER_22_2025.

## Conflict of interest

There are no conflicts to declare.

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