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(Article begins on next page)

1 **Whole blood and oral fluid microsampling for the monitoring**
2 **of patients under treatment with antidepressant drugs**

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22 **ABSTRACT**

23 Patients suffering from major depression and related pathologies (feeding and eating disorders, obsessive-
24 compulsive disorder, post-traumatic stress disorder, anxiety disorders, etc.) are usually treated with
25 antidepressant agents belonging to several pharmacological and chemical classes; the most recent of these
26 agents are collectively known as “new-generation antidepressants”. In these patients, therapeutic drug
27 monitoring (TDM) with the determination of drug and metabolite blood levels is one of the most useful
28 procedures to optimise and personalise the treatment, enhancing both effectiveness and safety. A new
29 approach is proposed in this study, based on microsampling of both blood and oral fluid by means of
30 volumetric absorptive microsampling (VAMS). This approach makes sampling and storage much simpler and
31 even self- and at-home-sampling possible, while retaining reliability, vastly increasing analyte stability and
32 reducing overall expenses. The microsamples were pretreated by means of microextraction by packed
33 sorbent (MEPS) on C2 sorbent and analysed by liquid chromatography with sequential spectrophotometric
34 and spectrofluorimetric detection (HPLC-UV-FL). Method validation results were satisfactory (extraction yield
35 >84%, precision RSD < 8.9%, stability >85.0% after 3 months). Application to blood and oral fluid VAMS from
36 patients treated with four possible different antidepressants (sertraline, fluoxetine, citalopram and
37 vortioxetine) provided results always in good agreement with those obtained from the corresponding fluid
38 matrices, including the levels of drug metabolites.

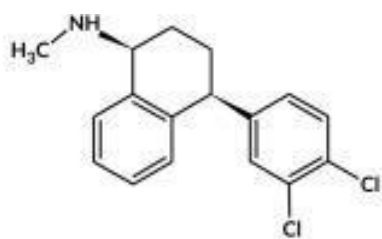
39
40 **Keywords**

41 Therapeutic drug monitoring (TDM); Microsampling; Volumetric absorptive microsampling (VAMS); Blood
42 Oral fluid; Antidepressants.

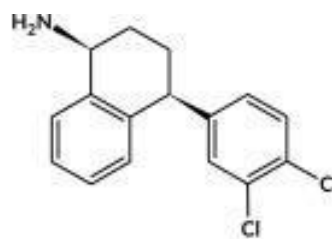
43 **1. INTRODUCTION**

44 According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) [1], major
45 depression is characterised by the nearly daily presence of symptoms like feeling of loneliness, sadness,
46 hopelessness; lack of energy; sleeping or eating disorders; thoughts of death or suicide. It is currently one of
47 the most frequent mental disorders after anxiety disorders [2]. Currently, the most effective forms of
48 treatment for depression involve, in addition to short-term goal-oriented psychotherapy, the use of
49 pharmacological antidepressant agents (ADA), and in particular the so-called “new-generation” ADA. These
50 drugs have demonstrated efficacy not only towards depression, but also against a range of related psychiatric
51 conditions, such as feeding and eating, obsessive-compulsive, post-traumatic stress and generalised or social
52 anxiety disorders. These drugs are divided into a plethora of different classes according to their specific
53 mechanism of action [3,4], while their common feature is their activity on (usually potentiation of) one or
54 more adrenergic amine pathways, and in particular those involving serotonin, norepinephrine and possibly
55 dopamine; melatonergic (agomelatine), glutamatergic (esketamine) and opioid (tianeptine) agents have also
56 been approved [5]. Another common feature of most ADA is their delayed onset of effect, that is usually about
57 6–12 weeks [6]. Unfortunately, in 20–30% of patients, current treatments are inadequate, and relapse is not
58 uncommon. No new-generation ADA has until now demonstrated to possess better efficacy and/or safety
59 for most patients than other agents [7], so the choice of the specific drug to be administered to each patient,
60 is mostly left to the specific expertise and preferences of the clinicians. In order to help psychiatrists in
61 correctly and objectively assessing the clinical situation and their therapeutic options, one of the most useful
62 practices is therapeutic drug monitoring (TDM) [8]. TDM provides for the periodic determination of drug and
63 metabolite plasma levels, together with the use of chemical-clinical correlations (i.e., correlations between
64 administered drug dose and plasma levels; between plasma levels and therapeutic efficacy; between plasma
65 levels and side and toxic effects) [21]. TDM can also lead to reduced healthcare expenses, due to the
66 possibility of better efficacy, increased patient compliance and enhanced safety, leading to a reduction in
67 hospitalisations due to unwanted effects or therapy ineffectiveness [9,10]. Dried microsampling can
68 substantially increase the feasibility and practicality of any TDM protocol. In fact, reducing the invasiveness
69 and complication of blood sampling is a good way to increase patient compliance (especially for psychiatric
70 patients that are often wary of any invasive procedure), possibly leading to widespread at-home self-
71 sampling practices [11]. Moreover, the loss of water usually increases analyte stability through reduction in
72 the rate of most degradation reactions. This in turn greatly reduces the precautions, appliances and space
73 needed for microsample storage and shipping in comparison to the corresponding fluid matrices [12]. In the
74 last few years, volumetric absorptive microsampling (VAMS) is attracting increasing interest, due to its

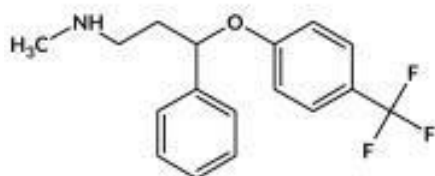
75 practicality, high sample volume reproducibility and, for blood-based microsamples, high independence of
76 sampling volume from haematocrit [13]: in fact, VAMS is carried out with a device including a calibrated
77 polymeric, porous tip that absorbs fixed volumes of matrix, according to its dimensions. It should be noted
78 that haematocrit differences can have other effects on analysis results, including changes in extraction yields
79 and precision, and matrix effect for mass spectrometric (MS) methods. VAMS-based analytical methods can
80 be subject to these sources of variability, even though sampling volume is not [14]. In addition to
81 microsampling, another attractive alternative practice is the use of non-invasive biological matrices instead
82 of blood, for example oral fluid (OF). OF includes all fluids present in the mouth, including saliva, oral mucosal
83 transudate, sputum from the lungs and throat, nasal drainage and others. It is one of the most promising non-
84 invasive matrices for TDM purposes, since its drug concentration can often closely mimic that of blood. Of
85 course, this needs to be verified case by case for each drug [15]. In this study, an analytical workflow is
86 described, based on volumetric absorptive microsampling (VAMS) of both blood and OF, followed by
87 microextraction by packed sorbent (MEPS) [16] and liquid chromatographic (HPLC) analysis with
88 spectrophotometric (UV) and spectrofluorimetric (FL) detection. The workflow has been validated for
89 application to the TDM of four different new-generation ADA: sertraline (SRT, Fig. 1a), fluoxetine (FLX, Fig.
90 1b), citalopram (CTP, Fig. 1c), vortioxetine (VTX, Fig. 1d) and their main metabolites: norsertaline (NSR, Fig.
91 1e), norfluoxetine (NFL, Fig. 1f), N-desmethylcitalopram (DCT, Fig. 1g) and N,N-didesmethylcitalopram (DDC,
92 Fig. 1h). As defined by recent Consensus Guidelines for TDM in neuropsychopharmacology, levels of
93 recommendation for TDM are: level 1 (strongly recommended) for CTP, level 2 (recommended) for SRT and
94 VTX, level 3 (useful) for FLX [13]. To the best of our knowledge, no scientific paper until now has been
95 published for the simultaneous determination of these four ADA and their main metabolites in biological
96 fluids. Regarding VTX in particular, just two papers have been published for its analysis in biological fluids
97 [17,18], but neither included any other ADA. As for the other, less recent new-generation ADA considered
98 herein, of course their analysis in biological fluids has been reported multiple times [19–23], but never using
99 the microsampling approach. In the ADA field, this approach has been proposed in just two papers: one for
100 the MS/MS screening of several psychiatric drugs in OF [24] and one for the screening of different drug classes
101 in dried urine spots (DUS) [25]. Neither paper includes all four considered drugs, nor application to blood-
102 based matrix microsamples.



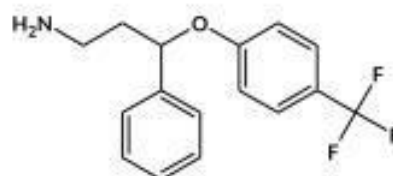
a – Sertraline (SRT)



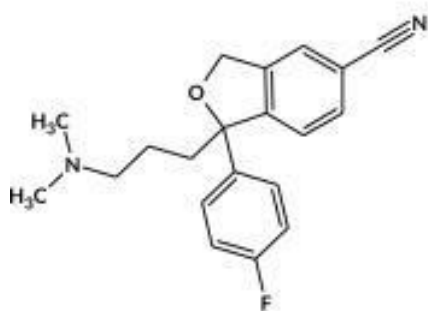
e – Norsertraline (NSR)



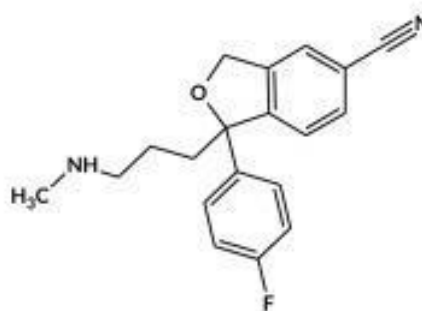
b – Fluoxetine (FLX)



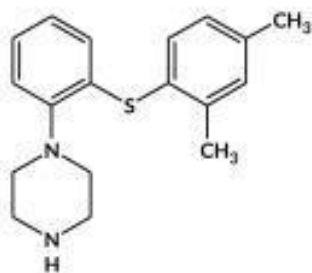
f – Norfluoxetine (NFL)



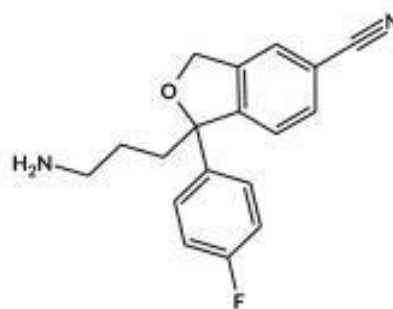
c – Citalopram (CTP)



g – N-desmethylcitalopram (DCT)



d – Vortioxetine (VTX)



h – N,N-didesmethylcitalopram (DDC)

Fig. 1. Chemical structures of (a) sertraline (SRT), (b) fluoxetine (FLX), (c) citalopram (CTP), (d) vortioxetine (VTX), (e) norsertraline (NSR), (f) norfluoxetine (NFL), (g) N-desmethylcitalopram (DCT) and (h) N,N-didesmethylcitalopram (DDC).

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107 2. MATERIALS AND METHODS

108 2.1. Chemicals and standard solutions

109 SRT((1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4- tetrahydronaphthalen-1-amine) hydrochloride, FLX (N-
110 methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) hydrochloride, CTP (1-[3-
111 (dimethylamino)propyl]-1-(4-fluorophenyl)-3H-2-benzofuran-5-carbonitrile) hydrobromide, NSR((1S,4S)-4-
112 (3,4-dichlorophenyl)-1,2,3,4- tetrahydronaphthalen-1-amine) hydrochloride, NFL (3-phenyl-3-[4-
113 (trifluoromethyl)phenoxy]propan-1-amine) hydrochloride, DCT (1-(4-fluorophenyl)-1-[3-
114 (methylamino)propyl]-3H-2-benzofuran-5-carbonitrile) hydrochloride, duloxetine (IS1, used in HPLC-UV for
115 VTX) hydrochloride and venlafaxine (IS3, used in HPLC-FL for CTP, FLX and metabolites) hydrochloride, pure
116 powders (all >99% purity); acetonitrile, methanol and dichloromethane (for HPLC, purity: > 99.9%),
117 monobasic potassium phosphate, triethylamine (TEA), phosphoric acid, sodium carbonate and potassium
118 hydroxide (all pure for analysis) were purchased from Sigma Aldrich Italy (Milan, Italy). VTX (1-[2-(2,4-
119 dimethylphenyl)sulfanylphenyl]piperazine) hydrobromide, DDC (1-(4-fluorophenyl)-1-[3-aminopropyl]-3H-2-
120 benzofuran-5-carbonitrile) hydrochloride and clotiapine (IS2, used in HPLC-UV for SRT and NSR) pure
121 powders were purchased from LGC Standards (Teddington, Middlesex, UK). Ultrapure water (18.2 M cm) was
122 obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). The analyte and IS stock
123 solutions (1 mg/mL) were prepared by dissolving suitable amounts of pure powders in methanol and kept at
124 -20 °C when not in use; the corresponding standard solutions were prepared daily by dilution with the HPLC
125 mobile phase. All solutions were stored protected from light in amber glass vials from Phenomenex
126 (Torrance, CA, USA).

127 128 2.2. HPLC-UV- FL instrumentation and conditions

129 HPLC-UV- FL analysis was performed on a Waters Corporation (Milford, MA, USA) Alliance e2695
130 chromatographic system with autosampler coupled to a Waters 2998 photo diode array detector and a Jasco
131 FP-2020 spectrofluorometric detector, connected in series. Separations were obtained on a Waters XBridge
132 BEH C18 column (150 × 2.1 mm, 3.5 μm) maintained at room temperature and equipped with a guard column.
133 The mobile phase was a mixture of 33 mM, pH 3.0 aqueous phosphate buffer containing 0.3% TEA (solvent
134 A) and acetonitrile (solvent B), flowing at a constant rate of 1.0 mL/min under gradient conditions. Gradient
135 composition was: 0.0–3.0 min, constant 20% B; 3.1–4.0 min, linear 20%–35% B gradient; 4.1–6.5 min,
136 constant 35% B; 6.6–7.5 min, linear 35%–55% B gradient; 7.6–14.5 min, constant 55% B; 14.6–15.5 min, linear
137 55%–20% B gradient, 15.6–17.0 min, constant 20% B to re-equilibrate the column. Both solvents were filtered on
138 a polyamide filter (47 mm diameter, 0.2 μm) and degassed by ultrasonication. Injection volume was 20 μL. SRT,

139 NSR and VTX, as well as IS1 and IS2, were detected by UV at 225 nm; FLX, CTP, NFL, DCT and DDC, as well as
140 IS3, were detected by fluorescence at $\text{em} = 235 \text{ nm}$, $\text{exc} = 300 \text{ nm}$.

142 **2.3. Real samples and compliance with ethical standards**

143 Real blood and oral fluid samples were obtained from in- and out-patients of different Psychiatric Clinics and
144 Hospitals of the Emilia-Romagna region in Italy, receiving antidepressants as part of their standard treatment
145 and were collected for general needs related to the therapy; all subjects provided informed consent prior to
146 their participation in this study. Sampling was carried out from patients in steady-state conditions and
147 atrough time, in the morning, at least 8 h (3 times a day dosing) or 12 h (twice a day dosing) after the previous
148 oral drug administration. In real samples, IS spiking was carried out on the VAMS tip by automatic pipetting
149 20 L of IS solution onto the tip before sampling; the tip was then left to dry for 2 h at RT before use.

151 **2.4. Sample pretreatment**

152 Mitra® VAMS microsamplers (20 L) were provided by Neoteryx (Torrance, CA, USA). A VAMS microsampler
153 includes a polypropylene handle (about 4 cm long) topped with a small tip (about 2-mm diameter) of a
154 proprietary polymeric porous material. B-VAMS. Blank or blank spiked B-VAMS were obtained by drawing a
155 few millilitres of blood from volunteers, then spiking it with the analytes and the ISs and accurately sampling
156 20 L of matrix by VAMS. 100-L blood aliquots were spiked with 5 L of analyte standard and/or IS mixtures at
157 known concentrations. The surface of the sample mixture was touched with a VAMS microsampler for 5 s,
158 dried at room temperature (RT) for 1 h and stored at RT in a dedicated clamshell in order to avoid contact
159 with any surface. VAMS microsamples were thus obtained. Clamshells were stored in sealed polyethylene
160 bags containing desiccant. For sample pretreatment, the microsampler tip was detached from the handle and
161 subjected ultrasound-assisted extraction (UAE) for 20 min in 1 mL of methanol. The resulting solution was
162 quantitatively transferred into a different vial and brought to dryness in a centrifugal evaporator. After re-
163 dissolving with 100 L of HPLC mobile phase (a 65:35 mixture of 33 mM, pH 3.0 aqueous phosphate buffer
164 containing 0.3% TEA / acetonitrile), the solution was subjected to MEPS pretreatment in an SGE Analytical
165 Science (Melbourne, VIC, Australia) C2 barrel-and-needle (BIN) assembly set up in an SGE eVol XR digital
166 analytical syringe apparatus. The BIN was activated by drawing and discarding 100 L of methanol 3 times and
167 conditioned with 100 L of water 3 times. The sample was loaded onto the BIN with 10 draw/discharge cycles
168 at a 5 L/s speed; the BIN was then washed twice with 100 L of water and 100 L of 10 mM, pH 9.0 carbonate
169 buffer / methanol (90/10, V/V) mixture at 20 L/s. The analyte and the ISs were eluted three times with 200 L
170 of methanol at 5 L/s (three cycles). After merging the three eluates, they were brought to dryness, re-

171 dissolved in 100 L of HPLC mobile phase (a 65:35 mixture of 33 mM, pH 3.0 aqueous phosphate buffer
172 containing 0.3% TEA / acetonitrile) and analysed by HPLC-UV-FL. OF-VAMS. Blank or blank spiked OF-VAMS
173 were obtained by drawing about 1 mL of OF from volunteers, then spiking it with the analytes and the ISs
174 and accurately sampling 20 L of matrix by VAMS. Oral fluid (1 mL) aliquots were centrifuged for 5 min at 6500
175 x g, then 100 L of supernatant were subjected to the same procedure as B-VAMS. ISs addition mode and
176 extraction performance assays (n = 6) were carried out by comparing pooled matrix fortification (as in blank
177 spiked samples) with VAMS tip ISs pre-soaking (as in real sample VAMS collection) in order to exclude any
178 bias in terms of volumetric accuracy and extraction efficiency.

179 **2.5. Method validation**

181 The analytical method was validated according to the International Conference on Harmonization of
182 Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [26] guidelines. The tested
183 parameters were linearity (including limits of detection and limit of quantitation), selectivity, extraction yield,
184 precision, carryover and accuracy. B-VAMS and OF-VAMS samples were spiked with analyte standard
185 solutions at seven different concentrations, containing the IS at a constant concentration, subjected to MEPS
186 pretreatment and injected into the HPLC system. The analysis was carried out in triplicate for each
187 concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding
188 concentrations (expressed as ng/mL) and the calibration curves were obtained by means of the least-square
189 method (1/x weighting). LOQ and LOD were calculated as the analyte concentrations, which gave rise to
190 peaks whose height was 10 and 3 times the baseline noise, respectively. For selectivity, six different blank
191 matrix samples from healthy volunteers were pretreated and analysed. In addition, some common CNS
192 drugs, such as chlorpromazine, clomipramine, clonazepam, clozapine, diphenhydramine, flurazepam,
193 fluvoxamine, fluphenazine, haloperidol, levosulpiride, lorazepam, loxapine, lurasidone, mirtazapine,
194 olanzapine, paroxetine, pipamperone, quetiapine, risperidone, trazodone, ziprasidone were injected in the
195 analytical system to check for selectivity. The obtained chromatograms were checked for interferences by
196 comparison with the peak area of the LOQ of each analyte, at their respective retention time. Selectivity was
197 considered acceptable if any extraneous peak was $\leq 20\%$ of the response of the LOQ of each analyte.
198 Extraction yields were evaluated by repeatedly subjecting to the previously described procedure blank
199 samples spiked with analyte standard solutions at four different, known concentrations (corresponding to
200 the LOQ, a low, an intermediate and a high value of the linearity range). The obtained analyte peak areas
201 were compared with those obtained by injecting standard solutions at the same theoretical concentrations
202 in order to calculate extraction yield values. Precision assays were carried out on the same samples, which

203 were analysed six times in the same day to obtain intraday precision and six times over six different days to
204 obtain interday precision, expressed as percentage relative standard deviation (RSD %). Carryover was
205 evaluated by injecting blank matrix extracts immediately after the highest calibration sample. The acceptance
206 criterion was no analyte peak higher than 20% of LOQ levels (5% for IS). Extraction performance assays with
207 respect to ISs addition mode were carried out by comparing spiking of fluid samples before VAMS sampling
208 (as in blank spiked samples) with VAMS tip presoaking (as in real VAMS sampling), in order to exclude any
209 bias in terms of extraction efficiency. To test analyte stability, microsamples were spiked with the analytes
210 at two concentration levels (high and low concentrations with respect to the calibration curve), then stored
211 at RT, protected from light, in sealed polyethylene bags containing desiccant for 3 months. At regular
212 intervals (1 week), microsamples were pretreated and analysed in triplicate. The measured analyte
213 concentrations were compared to those of the same samples extracted and analysed immediately after
214 microsampling and drying. The stability values thus obtained were also compared to those of fluid samples
215 stored at 4 °C, -20 °C or -80 °C. For autosampler processed stability, samples spiked at the same two
216 concentration levels were freshly pretreated in triplicate and stored in the autosampler at RT for 48 h before
217 re-analysis, while for bench-top stability, spiked, extracted microsamples were stored for 12 h at room
218 temperature without any further precautions. Samples were considered stable when % bias from the nominal
219 concentrations was within $\pm 15\%$. Recovery assays were carried out in order to evaluate method accuracy: 20
220 L of standard solutions containing known amounts of the analytes (corresponding to the LOQ, a low, an
221 intermediate and a high value of the calibration curves) were added to VAMS tips before microsampling real
222 samples. The obtained spiked samples were then analysed and analyte recovery was calculated by
223 comparison with non-spiked real samples.

2.6. Quantitative data comparisons

226 All results obtained from real samples were compared by plotting the results from each dried matrix (B-
227 VAMS, OF-VAMS,) versus those obtained from the corresponding fluid matrix analysis. Then, the least-square
228 method and Passing-Bablok regression were applied to calculate linearity correlation coefficient and slope of
229 each comparison curve, while Bland Altman plots were built to evaluate biases between results obtained
230 from microsamples and those obtained from fluid counterparts.

231 **3. RESULTS AND DISCUSSION**

232 **3.1. Chromatographic conditions optimization and IS choice**

233 For the development of chromatographic conditions, the starting point was isocratic elution systems
234 dedicated to the analysis of individual compounds together with their metabolites. A chromatographic
235 column was then selected to be tested under these conditions leading to the best performance in terms of
236 resolution and peak sharpness. The considered parameters were column length (50–200 mm), diameter (2.1–
237 4.6 mm) and sorbent particle size (1.7–5 μ m). The best compromise was achieved with a 150 mm length, 2.1
238 mm diameter, 3.5 μ m particle C18 column. For the optimisation of the mobile phase composition gradient,
239 several programs were carefully tested in terms of number of steps and slope, A/B solvents ratio and duration
240 of the single steps, to reach the best compromise between complete chromatographic resolution within the
241 two detection systems and total chromatographic run duration. Several drugs, not commonly used together
242 with the analytes in clinical settings, were tested as possible ISs. For example, in the HPLC-UV system
243 diphenhydramine, clotiapine, chlorpromazine, clomipramine, duloxetine, lamotrigine, loxapine and
244 indomethacine were tested; in the HPLC-FL system, mirtazapine, fluvoxamine, paroxetine and venlafaxine
245 were tested. Most compounds tested in the HPLC-UV system were detected within the testing run time
246 window; among these, loxapine, diphenhydramine, clomipramine and duloxetine had retention times not
247 overlapping with those of the analytes and not excessively long (to avoid unnecessary lengthening of total
248 run times). Since loxapine and diphenhydramine were scarcely retained, and based on structural
249 considerations, duloxetine (IS1) was chosen as the IS for VTX; clotiapine (IS2) was chosen as the IS for SRT
250 and its metabolite. Most compounds tested in the HPLC-FL system had unsuitable (either too strong or too
251 weak) retention, so the only suitable compound for both FLX and its metabolite, and CTP and its metabolites,
252 was deemed to be venlafaxine (IS3). Although the chosen ISs are CNS drugs, and in particular two
253 antidepressants and an antipsychotic agent, it should be noted that polypharmacy with different
254 antidepressants of the same generation (SSRIs, SNRIs, SMSs) is quite uncommon and clotiapine use is
255 uncommon in general, even more in association with antidepressants. Thus, interference due to
256 coadministration of an IS should be a very rare occurrence.

257

258 **3.2. VAMS procedure**

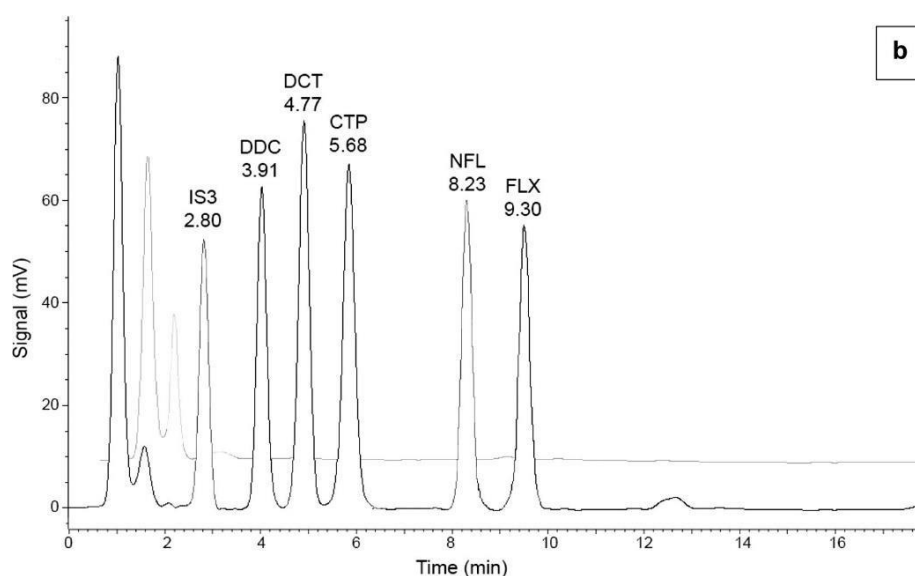
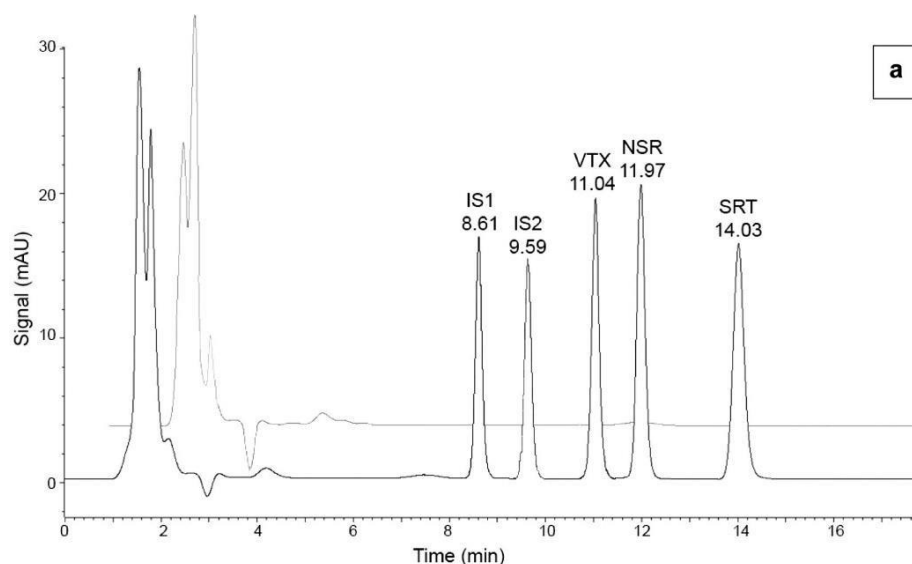
259 The biological matrix volume absorbed by VAMS tips has been studied for both B- and OF-VAMS. Six VAMS
260 devices for each matrix were weighed before and after sampling, then the accuracy and precision of sampling
261 volume were calculated. B-VAMS volume testing confirmed the high-volume accuracy (100.2%) and precision
262 (RSD = 4.7%) of this technique. 20-L OF-VAMS, on the contrary, have never been tested before, so the

263 following results are the first instance of such a procedure. OF-VAMS testing provided results that were
264 comparable to those obtained on B-VAMS: mean accuracy was 99.8% and RSD for precision was 5.7%. Sampling
265 time was also tested. Both B-VAMS and OF-VAMS provided basically constant sampling volumes over the
266 entire range of tested sampling times (1–20 s), excluding the shortest times (1, 2 and 3 s), which caused a
267 notably higher volume variability than higher sampling times (mean sampled volume < 75.5%, RSD > 15.9%).
268 Contact times of 10 and 20 s showed no over-sampling (mean volume accuracy 100.5%, RSD < 5.1%). Thus, 5
269 s was chosen as the sampling time that provided maximum speed while maintaining optimal reproducibility.
270 Gravimetric analysis on the time required to dry 20-L B-VAMS and OF-VAMS showed that under complete
271 ventilation at RT (25 °C; 55% humidity), the samples are dried within 1 h for B-VAMS and 45 min for OF-
272 VAMS, respectively. Extraction yield and volumetric comparative assays, performed by adding ISs to pooled
273 matrices before VAMS sampling and pre-soaking VAMS tips with ISs standard solution, provided satisfactory
274 and overlapping results (RSD < 4.1% as regards volumetric assays and < 5.0% for extraction yield), thus
275 demonstrating applicative suitability of both approaches.

276 277 **3.3. Sample pretreatment development**

278 VAMS can be thought as a pretreatment of sort, since the interactions between the analytes, the biological
279 matrix and the polymeric support can be exploited to elute the analytes selectively from the VAMS tip, or to
280 selectively eliminate matrix interferences. Several kinds of solvent extraction procedures were tested,
281 varying the kind of solvent (methanol, acetonitrile, ethyl acetate, diethyl ether, mixtures thereof, mixtures
282 with water or buffer), solvent volume (0.2–2.0 mL), contact time (1–10 min) and assistive technology
283 (microwaves, ultrasounds, vortex). Methanol provided better results than all other solvents and mixtures (i.e.
284 organic solvents provided better purification when compared to aqueous solutions, while methanol led to
285 higher extraction yields when compared to other organic solvents), and extraction yields increased with
286 extraction volumes up to 1 mL (e.g. mean extraction yield >84% with 1 mL and 84%). Using UAE, extraction
287 times provided increasing yields up to 20 min, so this was chosen as the best compromise. Despite the
288 notable interference decrease observed upon the optimised methanolic VAMS extraction, it was not
289 sufficient to obtain satisfactorily clean chromatograms (data not shown). As a consequence, a further
290 pretreatment step was applied, based on MEPS. MEPS is based on the same principles as SPE, but it is carried
291 out in a BIN containing minute amounts of sorbent and included into a syringe, using very small solvent
292 volumes. The procedure is carried out using an electronic automation device (eVol) that autonomously draws
293 and discharges known volumes of the chosen solvent at a known speed. All the main steps of the MEPS
294 procedure (loading, washing, elution) were optimized in order to obtain a satisfactory combination of

295 microsample purification and analyte extraction yields. For example, different solvent /cycle /speed
296 combination were tried. It was found that loading and elution provide good yields when they are carried out
297 at low speed (5 L/s) to grant strong analyte interaction with the sorbent and the eluent, respectively; on the
298 contrary, washing is best carried out at higher speed (20 L/s) to avoid unnecessary analyte loss. Similarly,
299 loading was complete after at least 10 cycles, with fewer cycles not granting high yields (mean extraction
300 yield <63% with 5 cycles); washing with hydrophilic and basic solvents (water, carbonate buffer/methanol,
301 respectively) correctly retained the analytes while eliminating most interference. Regarding elution,
302 methanol provided optimal analyte solubility and thus good yields; mixtures with either water, buffer or
303 acetonitrile, even at low ratios, decreased this parameter (mean extraction yield <72%). Under the final
304 microsampling and sample pretreatment conditions, an HPLC-UV chromatogram of a blank B-VAMS sample
305 spiked with SRT, NSR, VTX and their ISs appears as shown in Fig. 2a; an HPLC-FL chromatogram of a blank B-
306 VAMS sample spiked with FLX, NFL, CTP, DCT, DDC and their IS is shown in Fig. 2b. Both chromatograms are
307 superimposed to blank sample chromatograms, highlighting satisfactory method selectivity. Chromatograms
308 of OFVAMS counterparts are reported in Supplementary Material(Figures SF1a and SF1b, respectively).



309
 310 **Fig. 2.** (a) HPLC-UV chromatogram of a blank B-VAMS sample spiked with SRT, NSR, VTX and their ISs,
 311 superimposed to a blank B-VAMS sample and (b) HPLC-FL chromatogram of a blank B-VAMS sample spiked
 312 with FLX, NFL, CTP, DCT, DDC and IS, superimposed to a blank B-VAMS sample.
 313

314 3.4. Method validation

315 3.4.1. Linearity

316 Concentration ranges for linearity assessment were selected on the basis of the expected concentration
 317 ranges and by taking into account back-calculated concentrations when performing linearity assays
 318 (acceptance criteria was defined as $\pm 15\%$ of the nominal value). Good linearity was obtained for both dried
 319 matrices and all analytes, with r^2 values always higher than 0.9989 and up to 0.9998; neither of the two
 320 matrices produced better linearity results than the other. Sensitivity values were generally better for OF-

VAMS than B-VAMS due to slightly better signal-to-noise ratios, while LOQ results were compatible with an effective monitoring of the considered drugs and their metabolites (see Table 1).

Table 1. Linearity, LOQ, LOD on spiked samples.

Analyte	Matrix	Linearity range, ng/mL	r^2	LOQ, ng/mL	LOD, ng/mL
SRT	B-VAMS	7–500	0.9995	7.0	2.5
	OF-VAMS	5–500	0.9993	5.0	1.5
FLX	B-VAMS	10–750	0.9990	10.0	3.0
	OF-VAMS	7–750	0.9992	7.0	2.5
CTP	B-VAMS	1–200	0.9997	1.0	0.3
	OF-VAMS	1–200	0.9997	1.0	0.3
VTX	B-VAMS	5–500	0.9994	5.0	1.5
	OF-VAMS	3–500	0.9992	3.0	1.0
NSR	B-VAMS	7–500	0.9990	7.0	2.5
	OF-VAMS	5–500	0.9993	5.0	1.5
NFL	B-VAMS	10–750	0.9991	10.0	3.0
	OF-VAMS	7–750	0.9992	7.0	2.5
DCT	B-VAMS	1–200	0.9996	1.0	0.3
	OF-VAMS	1–200	0.9998	1.0	0.3
DDC	B-VAMS	1–200	0.9993	1.0	0.3
	OF-VAMS	1–200	0.9995	1.0	0.3

3.4.2. Extraction yield, precision

The results of extraction yield and precision assays are reported in Table 2. Extraction yields were quite good, in the 86–95% range for B-VAMS and in the 85–96% for OF-VAMS, while precision, expressed as RSD values, was always in the 5.4–8.8% range. Comparative extraction yield assays, performed by adding ISs to fluid matrices before VAMS sampling and pre-soaking VAMS tips with IS standard solution, provided overlapping results (extraction yield range 85–95%, RSD < 6.8%), thus demonstrating the suitability of both approaches.

335 **Table 2.** Extraction yield and precision in spiked samples.

Analyte	Concentration level ^a	Matrix	Extraction yield, % ^b	Precision, RSD% ^c	
				Intraday	Interday
SRT	LOQ	B-VAMS	89	6.7	8.1
		OF-VAMS	90	7.0	7.9
	Low	B-VAMS	91	6.5	7.9
		OF-VAMS	91	6.7	7.7
	Intermediate	B-VAMS	92	6.0	7.5
		OF-VAMS	93	6.1	7.2
High	B-VAMS	93	5.8	7.0	
	OF-VAMS	95	6.0	6.7	
FLX	LOQ	B-VAMS	90	6.9	7.8
		OF-VAMS	91	7.5	8.4
	Low	B-VAMS	91	6.4	7.6
		OF-VAMS	92	7.4	8.0
	Intermediate	B-VAMS	93	5.9	7.3
		OF-VAMS	93	7.2	7.6
High	B-VAMS	94	5.6	6.8	
	OF-VAMS	96	5.8	6.9	
CTP	LOQ	B-VAMS	89	6.2	7.9
		OF-VAMS	91	7.0	8.1
	Low	B-VAMS	90	5.9	7.6
		OF-VAMS	91	7.7	7.9
	Intermediate	B-VAMS	92	5.7	7.2
		OF-VAMS	93	6.0	7.5
High	B-VAMS	95	5.4	6.6	
	OF-VAMS	95	5.8	6.9	
VRX	LOQ	B-VAMS	87	7.0	8.2
		OF-VAMS	89	7.2	8.4
	Low	B-VAMS	88	6.8	7.8
		OF-VAMS	90	7.2	7.6
	Intermediate	B-VAMS	90	6.1	7.6
		OF-VAMS	90	6.9	7.4
High	B-VAMS	91	5.6	7.0	
	OF-VAMS	93	6.1	7.2	
NSR	LOQ	B-VAMS	87	7.3	8.6
		OF-VAMS	85	7.3	8.4
	Low	B-VAMS	88	6.9	8.4
		OF-VAMS	88	7.0	7.0
	Intermediate	B-VAMS	88	6.4	8.0
		OF-VAMS	91	6.6	7.6
High	B-VAMS	90	5.9	7.4	
	OF-VAMS	92	5.9	7.1	
NFL	LOQ	B-VAMS	87	7.5	8.6
		OF-VAMS	88	7.2	8.8
	Low	B-VAMS	87	7.4	8.3
		OF-VAMS	89	6.9	8.3
	Intermediate	B-VAMS	89	7.0	7.9
		OF-VAMS	89	6.8	7.8
High	B-VAMS	91	5.8	7.3	
	OF-VAMS	91	5.6	7.4	
DCT	LOQ	B-VAMS	87	7.6	8.8
		OF-VAMS	85	7.4	8.7
	Low	B-VAMS	87	7.5	8.6
		OF-VAMS	86	7.2	8.2
	Intermediate	B-VAMS	88	7.3	8.0
		OF-VAMS	87	6.9	7.7
High	B-VAMS	89	6.0	7.5	
	OF-VAMS	90	6.1	6.9	
DDC	LOQ	B-VAMS	86	7.7	8.5
		OF-VAMS	85	7.7	8.6
	Low	B-VAMS	86	7.0	8.2
		OF-VAMS	88	7.5	8.4
	Intermediate	B-VAMS	88	6.6	7.6
		OF-VAMS	88	6.9	7.8
High	B-VAMS	90	5.8	6.8	
	OF-VAMS	91	6.2	7.2	

336

337

^a For each matrix, “Low”, “Intermediate” and “High” concentrations are referred to the respective linearity curve.

338

^b n = 6, mean value. ^c n = 6.

3.4.3. Selectivity and carryover

No interfering peak higher than 20% of the response of the LOQ of each analyte was detected in any of the blank samples, and no interfering peak at the retention time of the analytes or ISs under the two detection means was also observed when injecting into the HPLC system common CNS drugs. Moreover, carryover was within the defined acceptance criteria: interference signals were < 20% of the LOQ (<5% for ISs).

3.4.4. Stability

Dried microsamples are usually stored at RT while maintaining good stability, thanks to the lack of water that effectively stops many chemical and enzymatic reactions, and also causes molecular immobilisation. In this case, analyte stability in spiked B-VAMS and OF-VAMS after 3 months resulted to be very high: mean stability was > 88.1% (range: 88.2–97.2%) for B-VAMS and >85.0% (range: 85.1–94.8%) for OF-VAMS. Moreover, all analytes proved to be stable (>85.3%) also in all remaining stability assays (autosampler processed and bench-top assays).

3.5. Analysis of real samples and accuracy

Having validated the microsampling, pretreatment and analysis workflow, real samples from psychiatric patients undergoing treatment with new generation antidepressants were analysed. Examples of chromatograms of OF-VAMS samples obtained from patients treated with SRT (75 mg/d) and FLX (50 mg/d) are shown in Fig. 3a and b, respectively. As one can see, analyte separation is similar to that obtained on the corresponding spiked blank samples. Table 3 shows the B-VAMS and OF-VAMS data obtained from real patient samples. As can be seen, the administered drug was identifiable and quantifiable in all B-VAMS and OF-VAMS samples, together with the considered metabolites. As for matrix comparison, no clear correlation was found, with OF-VAMS results usually much lower than those of B-VAMS except for VTX, with OF-VAMS concentrations slightly higher than those observed in B-VAMS. In order to evaluate the analytical performance of the proposed dried microsampling approaches, the results obtained from VAMS on real samples (Table 3) were compared to those obtained from fluid matrix counterparts (plasma and OF) by using fully validated procedures (Supplementary Material S1) and after converting plasma results to whole blood concentrations by using mean erythrocyte-to-plasma concentration ratios for the target analytes [27–30]. Sample pretreatment was based on solid phase extraction (SPE) for plasma and on liquid-liquid extraction (LLE) for OF, both coupled to HPLC-UV-FL analysis. B-VAMS results are always very similar to those of the original fluid matrix, i.e., plasma concentrations after conversion to whole blood values (data not shown). Bland-Altman differences plots were built for B-VAMS/plasma and OF-VAMS/fluid OF correlations (Fig. 4a

371 and b, respectively) together with scatter plots (Supplementary Material Figure SF2). These graphs and
 372 Passing-Bablok regression, testify as to the agreement between each miniaturised matrix and its
 373 corresponding fluid matrix. Passing-Bablok regression produced for B-VAMS - plasma comparison (n = 16) a
 374 slope coefficient of 1.0054 (95% CI, 0.9652–1.0388), an intercept of 0.2512 ng/mL (95% CI, –1.3305 ng/mL–
 375 1.5911 ng/mL) and an r2 value of 0.9964; for OF-VAMS - fluid OF comparison (n = 16), slope coefficient was
 376 of 1.0000 (95% CI, 0.9135–1.0746), intercept was 0.2500 ng/mL (95% CI, –1.5500 ng/mL–2.7596 ng/mL) and
 377 r2 was 0.9941. LOQ values for the microsampling methods were about twice those of the corresponding fluid
 378 matrix methods, despite the former ones diluting the samples 5 times more than the latter ones. This can be
 379 at least in part attributed to the better sample purification level achieved by VAMS coupled to MEPS
 380 pretreatment. Accuracy assays provided very high recovery values, in the 86–94% range for B-VAMS and 88–
 381 93% for OF-VAMS. In conclusion, both microsampling methods provide reliable analytical results. The results
 382 obtained from B-VAMS can be directly applied for TDM purposes. For those obtained from OF-VAMS, no
 383 clear correlation was observed with those of whole blood, with generally lower concentrations in the former
 384 one, but of course more data are needed to evaluate VAMS-OF as a useful matrix for TDM.

385

386 **Table 3.** Patient sample analysis.

Subject n.	Treatment, dose (mg/d)	Biological matrix	Concentration found, ng/mL ^a		
			Parent drug ^b	Metabolite 1 ^c	Metabolite 2 ^d
1	SRT, 75	B-VAMS	41.3	53.2	–
		OF-VAMS	32.6	27.9	–
2	SRT, 100	B-VAMS	88.6	116.1	–
		OF-VAMS	48.6	36.5	–
3	FLX, 50	B-VAMS	49.8	48.3	–
		OF-VAMS	26.8	30.4	–
4	FLX, 60	B-VAMS	86.9	127.1	–
		OF-VAMS	41.6	57.9	–
5	CTP, 20	B-VAMS	54.9	26.9	7.5
		OF-VAMS	36.4	13.4	4.8
6	CTP, 30	B-VAMS	100.6	52.1	14.7
		OF-VAMS	53.6	23.8	6.6
7	VTX, 7.5	B-VAMS	9.6	–	–
		OF-VAMS	10.4	–	–
8	VTX, 10	B-VAMS	13.3	–	–
		OF-VAMS	17.8	–	–

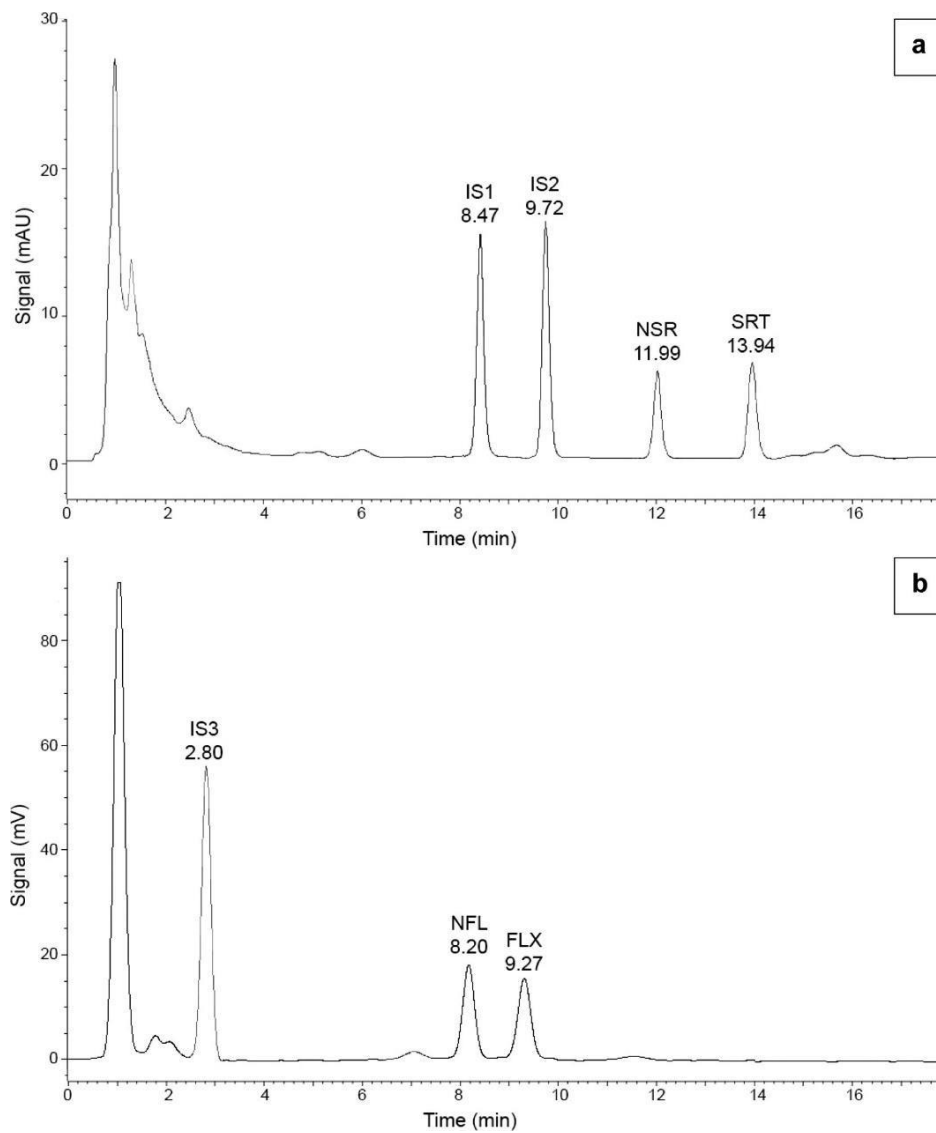
387

388 a n = 3.

389 b The same drug indicated in the “treatment” column.

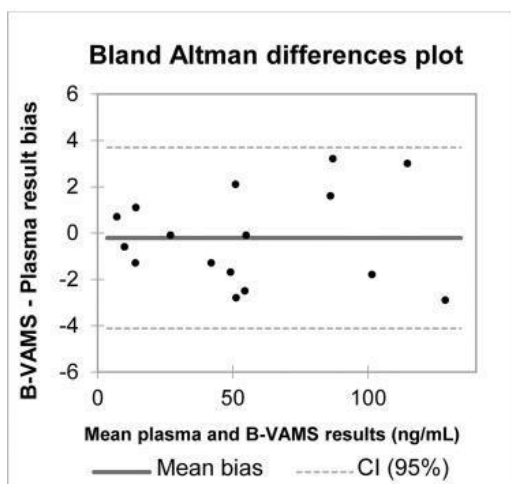
390 c NSR for patients treated with SRT; NFL for patients treated with FLX; DCT for patients treated with CTP.

391 d DDC for patients treated with CTP.

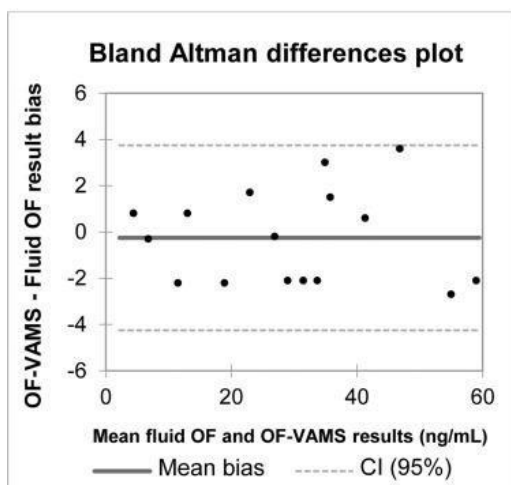


392 **Fig. 3.** (a) HPLC-UV chromatogram of an OF-VAMS sample obtained from a patient treated with SRT (75
 393 mg/d) and (b) HPLC-FL chromatogram of an OF-VAMS sample obtained from a patient treated with FLX (50
 394 mg/d).
 395

a - Plasma vs. B-VAMS results



b - Fluid OF vs. OF-VAMS



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Fig. 4. Bland Altman differences plots for the following result comparison: plasma vs. B-VAMS (a) and fluid OF vs. OF-VAMS (b). Plasma results are intended after conversion to whole blood values by means of erythrocyte-to-plasma concentration ratios for each analyte.

401 **4. CONCLUSION**

402 An analytical workflow, based on B-VAMS or OF-VAMS, MEPS pretreatment and HPLC-UV-FL determination,
403 has been developed, validated and applied to the TDM of patients suffering from major depression and/or
404 related disorders, treated with SRT, FLX, CTP or VTX. Microsampling volume by VAMS for both blood and oral
405 fluid was tested, and good data were obtained (volume accuracy 100.2%, precision RSD 4.7% for B-VAMS and
406 volume accuracy 99.8%, precision RSD 5.7% for OF-VAMS). Validation results were very satisfactory, with
407 high extraction yields (>85% for B-VAMS and >84% for OF-VAMS) and accuracy (>85% and >87% for B-VAMS
408 and OFVAMS, respectively), and low RSD values for precision (85.0% for all analytes and all matrices, even
409 though B-VAMS and OF-VAMS were stored at RT. Finally, good agreement was obtained between the data
410 obtained from the TDM of patients when using B-VAMS as opposed to blood and OF-VAMS as opposed to
411 oral fluid. However, no clear, convincing correlation between blood and OF levels was found for any of the
412 considered drugs. Of course, the analysis of many more patient samples is needed for this purpose. In
413 conclusion, both B-VAMS and OF-VAMS seem to be suitable for the reliable and feasible analysis of blood
414 and OF levels, respectively, of antidepressants in psychiatric patients. Regarding at-home self-sampling
415 specifically, this study represents a first proof-of-concept of the possible application of VAMS for TDM
416 purposes. In fact, VAMS sampling can easily be carried out by most people without any particular training,
417 let alone a specific healthcare training. Obviously, more extensive experimentation and statistical evaluations
418 should be carried out on large numbers of volunteers, including the acceptability and preferences of patients,
419 as well as the possible effects of self-sampling on TDM reliability and accuracy.

420

421 **Declaration of Competing Interest**

422 The authors declare that they have no known competing financial interests or personal relationships that
423 could have appeared to influence the work reported in this paper.

424

425 **CRedit authorship contribution statement**

426 Camilla Marasca: Methodology, Investigation, Formal analysis, Validation, Writing - original draft. Michele
427 Protti: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing,
428 Visualization. Roberto Mandrioli: Conceptualization, Resources, Data curation, Writing - original draft,
429 Writing - review & editing. Anna Rita Atti: Writing - review & editing, Supervision, Resources, Funding
430 acquisition. Andrea Armirotti: Writing - review & editing, Resources. Andrea Cavalli: Resources, Funding
431 acquisition. Diana De Ronchi: Writing - review & editing, Resources, Funding acquisition, Project

administration. Laura Mercolini: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition, Project administration.

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