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1 **Development of a new in-line coupling of a miniaturized boronate affinity monolithic column with**
2 **reversed-phase silica monolithic capillary column for analysis of cis-diol-containing nucleoside**
3 **compounds**

4
5 Maria Betzabeth Espina-Benitez, Jérôme Randon, Claire Demesmay, Vincent Dugas*

6
7 *Université de Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, UMR 5280, 5 rue*
8 *de la Doua, F-69100 VILLEURBANNE, France*

9 * Corresponding author. Tel. : +33437423552

10 E-mail address: Vincent.dugas@univ-lyon1.fr

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14

15 **Abstract**

16 In-line coupling of capillary columns is an effective means for achieving miniaturized and
17 automated separation methods. The use of multimodal column designed to allow the direct
18 integration of a sample preparation step to the separation column is one example. Herein we
19 propose a novel in-line coupling at the capillary scale between a boronate affinity capillary
20 column (μ BAMC unit) and a reversed-phase separation column. This has been made possible
21 due to the elaboration of a new and efficient μ BAMC unit. A thiol-activated silica monolithic
22 capillary column was functionalized through thiol-ene photoclick reaction. This simple and
23 fast reaction allows to prepare stable μ BAMC units having grafting densities of 1.93 ± 0.17
24 nmol cm^{-1} . This grafting strategy increases the surface density by a factor 4 compared to our
25 previous strategies and opens the frame to in-line coupling with reversed-phase capillary
26 column. Proof of concept of the in-line coupling was done by coupling a 1-cm length
27 μ BAMC unit to a 7-cm length reversed phase capillary column. The conditions of loading,
28 elution and separation were optimized for cis-diol nucleosides analysis (uridine, cytidine,
29 adenosine, guanosine). A loading volume (at pH 8.5) of up to 21 hold volume (i.e $1\mu\text{l}$) of the
30 μ BAMC unit can be loaded without sample breakthrough. For the least retained nucleoside
31 (uridine) a limit of detection of 50 ng mL^{-1} was estimated. Elution and full separation of the
32 four nucleosides was triggered by flushing the multimodal column with an acetic acid
33 (50mM) / methanol (98/2, v/v) mobile phase.

34

35

36 Keywords: multimodal chromatography, photoclick chemistry, in-line coupling, boronate
37 affinity, capillary chromatography.

38

39 1. Introduction

40 Miniaturized analytical methods are attractive in the context of waste and cost reduction,
41 automation, in-situ / handheld analysis and personalized medicines. This non-exhaustive list
42 shows the great diversity of applications that are in demand. In such frame, separation
43 sciences are also part of this process. To go beyond the straightforward column reduction, in-
44 line coupling of the sample preparation step to the separation column is proposed. In-line
45 coupling of a sample preparation step allows not only to purify but also to preconcentrate
46 analytes. This is key feature regarding sensibility problem associated to miniaturization. In
47 addition, in-line couplings are “automated by-design”.

48 However, relevance of such in-line coupling depends on (i) the binding capacity of the
49 purification/preconcentration zone (ii) the ability to elute the captured fraction in a small plug
50 of solution whose composition has also to be adapted to the following separation step
51 (mechanism). Finding appropriate conditions to meet these requirements is challenging and
52 may account for the relatively limited number of reported in-line coupling combinations.
53 Classically, miniaturized in-line couplings implement purification steps based on strong
54 interactions mechanisms (i.e. ion exchange, immunoaffinity) coupled to reversed-phase
55 analyses. Indeed, the purification and elution steps are done under aqueous media and the
56 elution plug directly transferred to the reversed-phase column.

57 Herein we investigated a novel in-line coupling of a boronate affinity
58 purification/preconcentration step to a reversed-phase separation. Boronate affinity
59 chromatography (BAC) has unique affinity towards cis-diol containing compounds that cover
60 a wide range of molecules of interest in pharmaceutical and biomedical research
61 (catecholamines, glycoproteins, RNA, etc) [1,2] and supports the use of salt and organic
62 solvent [3]. This reversible reaction allows the capture of cis-diol compounds under basic
63 conditions and their rapid release under acidic media. Miniaturized boronate affinity
64 monolithic unit (μ BAMC) were in-line coupled to electrokinetic separations[4,5]. While only
65 on-line coupling of boronate affinity column to reversed-phase liquid chromatography has
66 already been reported [6–10] highlighting the pertinence of such coupling, no
67 chromatographic in-line couplings were developed. Analysis of complex mixtures at trace levels
68 has pushed forward the development of integrated and miniaturized separation systems, coupling
69 the preconcentration and separation steps. Besides the “off-line” classical approach, two different
70 coupling approaches, so-called “on-line” or “in-line” couplings, have been proposed in the literature.

71 The on-line approach is referred as the physical coupling of the two distinct preconcentration and
72 separation columns, through specific interfaces like 10-port valves. Although this approach has
73 benefits in terms of automation, the instrumentation set-up is usually complex and band broadening
74 consecutive to the physical coupling is critical in miniaturized systems [11]. The in-line mode
75 represents the highest level of integration and automation and is well adapted for nano-separations.
76 The μ -SPE unit, located at the inlet of the capillary column, is an integral part of it [12]. In such
77 systems, adapted to microliter sample volumes, the elution of the μ -SPE unit is achieved with a very
78 low volume of solution (few tens of nanoliters) that is sharply directed to the separation zone,
79 without dilution or band broadening effects. Our objective is to demonstrate the feasibility of a
80 miniaturized in-line coupling of monolithic boronate affinity column to reversed-phase
81 monolithic capillary column. We describe the preparation of μ BAMC units using a thiol-ene
82 photoclick reaction, which allows a 4-fold increase in the number of phenylboronate sites
83 compared to photopolymerization reaction. This is of paramount importance to balance the
84 relatively low affinity of cis-diol compounds for boronate affinity column. Conditions of in-
85 line coupling of such preconcentration units were investigated. Finally, a proof of feasibility
86 of the in-line coupling was done by coupling a 1-cm length μ BAMC unit with a 7-cm length
87 reversed-phase monolithic column.

88

89 **2. Materials and methods**

90 *2.1 Reagents and chemicals*

91 All the reagents used were of analytical grade. Tetramethoxysilane (TMOS),
92 methytrimethoxysilane (MTMS), urea, polyethylene glycol (PEG, MW =10,000), 3-
93 (acrylamido)phenylboronic acid (AAPBA), triethylamine (TEA), methanol (MeOH, HPLC
94 grade), 1,2-dihydroxybenzene (catechol), 1,4-Benzenediol (hydroquinone), adenosine, 2'-
95 deoxyadenosine, uridine, cytidine, guanosine, thiourea, 3-(mercaptopropyl)trimethoxysilane
96 (MPTMS), benzophenone, Na_2HPO_4 , NaH_2PO_4 were purchased from Sigma-Aldrich (Sigma-
97 Aldrich, Saint-Quentin-Fallavier, France). n-Octadecyl dimethyl(dimethylamino)silane was
98 from Gelest inc. (Germany). Acetonitrile (ACN) (HPLC grade) was purchased from VWR
99 (VWR, Fontenay-sous-bois, France). Acetic acid (AcOH) was from Riedel-de Haënn (Riedel-
100 de Haënn, Seelze, Germany). All aqueous solutions were prepared using $>18 \text{ M}\Omega\text{-cm}$ water
101 (Millipore, Molsheim, France). PTFE-coated fused-silica capillaries (TSU deep-UV

102 Transparent Coating 75 μm i.d.) were purchased from Polymicro Technologies (Polymicro
103 Technologies, Arizona, USA).

104 *2.2 Instrumentation*

105 Nano-LC experiments were carried out with an Agilent HP3D CE system (Agilent
106 Technologies, Waldbronn, Germany) equipped with a diode array detector and exclusively
107 used in the pressurization mode with an external pressure device allowing to work up to 12
108 bars (no voltage applied). System control and data acquisition were carried out using the
109 Chemstation software (Agilent). In-line coupling was realized by physically coupling
110 μBAMC unit to RP-monolithic column using fluorinated ethylene-propylene tubing (FEP
111 tubing 330 μm i.d. x 1.55 mm o.d. (Cluzeau info labo, Sainte-Foy-La-Grande, France) (see
112 Figure S1).

113 *2.3 Preparation of functionalized silica monoliths*

114 *2.3.1. Synthesis of silica monoliths*

115 The silica monolith preparation followed our reported procedure [13]. Briefly, a 18 mL
116 mixture of TMOS/MTMS (85/15 v/v) was added to 40 mL of 0.01 M acetic acid solution
117 containing 1.9 g PEG and 4.05 g urea. The sol mixture was stirred at 0°C for 30 min. Then,
118 the temperature was raised to 40°C and the mixture was loaded into the 75- μm fused-silica
119 capillary and kept overnight at 40°C for gelification completion. Mesopores were formed by
120 urea decomposition gently raising the capillary temperature at 0.5°C min^{-1} up to 120°C
121 maintained for 4 hours. After cooling, the monolith was washed with methanol and the
122 capillary was cut to desired length.

123

124 *2.3.2. C18-functionalization of silica monoliths*

125 10-cm length monolithic capillary columns were filled with pure n-Octadecyl
126 dimethyl(dimethylamino)silane and heated at 110°C for 2 hours (adapted from [14]).
127 Capillary columns were then thoroughly rinsed with THF.

128

129 *2.3.3. AABPA-functionalization of silica monoliths*

130 *- Thiol pre-functionalization*

131 Prior to surface modification by photo-click chemistry, silica monoliths were
132 prefunctionalized with 3-(mercaptopropyl)trimethoxysilane (MPTMS) to anchor thiol
133 moieties onto the surface by covalent bonding. The silanization solution was composed of

134 MPTMS (5% v/v) in toluene. Silanization was performed at 37°C during 3 hours under
135 hydrodynamic flow and then cured at 100°C overnight. After silanization, monoliths were
136 thoroughly rinsed with THF and MeOH.

137 - “Thiol-ene” photo-click functionalization of monoliths

138 The thiol pre-functionalized silica support was filled with a solution containing a
139 photoinitiator (12.5 mM benzophenone) and the 3-(acrylamido)phenylboronic acid monomer
140 (100 mM AAPBA) in an aqueous TEA (pH 9)/acetonitrile mixture (50/50, v/v). Then, the
141 ends of the capillary were plugged with rubber and the capillary irradiated for 10 min at 365
142 nm. The illumination system used for photo-click reaction was a BioLink cross linker (VWR
143 Inter-national, Strasbourg, France) equipped with five 8 W UV tubes emitting at 365 nm. The
144 capillary was then washed extensively with a water / acetonitrile (50/50 v/v) mixture and then
145 with methanol. In some cases, several successive graftings (from 3 to 10 as indicated in the
146 text) were implemented after refreshing the grafting solution inside the capillary in-between
147 each irradiation step. A schematic representation of the AAPBA-functionalization is presented
148 in Figure S2.

149 *2.3 Determination of the number of actives sites and cis-diol affinity (Kd) by in-capillary*
150 *frontal analysis* [15]:

151 A 1-3-cm length silica monolithic segment at the inlet of a 10-cm length fused silica capillary
152 was used to determine the total number of actives sites (B_{act}) on boronate affinity monolithic
153 columns by frontal analysis. Catechol was used as cis-diol model molecule to probe the
154 photografting reaction. Indeed, catechol is widely used in boronate affinity materials to
155 characterize the number of actives sites due to its relatively high affinity with phenylboronate
156 ($K_d \sim 300 \mu\text{M}$). Catechol interacts with phenylboronate groups through specific cis-diol
157 interactions to form 5-membered ester rings under basic conditions. Hydroquinone was used
158 to evaluate the specificity of the μBAMC . Indeed, hydroquinone is a positional isomer (non
159 cis-diol) with comparable non-specific interaction than catechol with the solid support.
160 Breakthrough curves (detection at 270 nm) with catechol as model solute at 100, 300 and 500
161 $\mu\text{g mL}^{-1}$ in phosphate buffer (15 mM Na_2HPO_4 , pH 8.5)/MeOH (80/20, v/v) were used to
162 determine the amounts of catechol (q) captured at different catechol concentrations [L]
163 according to equation 1:

164

165

$$q = \Delta t \times F \times [L] \quad (\text{Eq. 1})$$

166 F being the flow rate and Δt being the time difference between dead time, t_0 , and saturation
167 time, t_{plateau} . The total number of active sites on the affinity column, B_{act} , and the dissociation
168 equilibrium constant (K_d) were determined according to equation (2) after plotting $1/q$ versus
169 $1/[L]$ (linear plot):

170

$$\frac{1}{q} = \frac{K_d}{B_{act}} \times \frac{1}{[L]} + \frac{1}{B_{act}} \quad (\text{Eq. 2})$$

171 2.4 Determination of the loading capacity and LOD

172 After preconditioning the column with phosphate buffer at pH 8.5, an injection volume of 50
173 nL of uridine solution (corresponding to 1.2 volume of the μBAMC unit) was loaded on the
174 multimodal nano-column. The interstitial liquid was then removed from the μBAMC unit by
175 percolating 42 nL of phosphate buffer pH 8.5 (corresponding to 1 volume of μBAMC unit).
176 This step allowed to remove unretained compounds from the μBAMC unit before the final
177 elution step. The μBAMC was eluted by flushing the multimodal column with a 50 mM acetic
178 acid solution. This experiment was repeated with increasing concentration of uridine (from 1
179 to 100 $\mu\text{g mL}^{-1}$). All steps were conducted at a constant pressure of 12 bars (i.e. a linear
180 velocity of 0.16 mm s^{-1}).

181 The limit of detection (LOD) was determined by using a capillary column composed of a 1-
182 cm μBAMC monolith coupled to a 7-cm length reversed-phase column. Solutions of uridine
183 solubilized in the loading buffer (15 mM phosphate solution (pH 8.75)) were loaded through
184 the column at a constant pressure of 12 bars (i.e. a linear velocity of 0.16 mm s^{-1}) for
185 increasing duration depending on the volume to be loaded. The interstitial liquid was then
186 removed from the μBAMC unit by percolating 42 nL of phosphate buffer pH 8.5
187 (corresponding to 1 volume of μBAMC unit). The elution and separation steps were
188 performed using a 50 mM acetic acid mobile phase.

189

190

191 3. Results and discussion

192 3.1 Thiol-ene versus Ene-thiol photoclick grafting of AAPBA

193 We previously detailed the *in-situ* preparation of two phenylboronate affinity capillary
194 columns (75 μm i.d.) : a porous polymer monolith used to work under harsh pH conditions [5]
195 and a silica-based monolith [4], characterized (using catechol) by a number of phenylboronate
196 sites of $0.16 \text{ nmol cm}^{-1}$ ($3.6 \text{ nmol } \mu\text{L}^{-1}$) and $0.43 \text{ nmol cm}^{-1}$ ($9.7 \text{ nmol } \mu\text{L}^{-1}$), respectively. In
197 order to further increase the boronate affinity capacity of silica monoliths i.e to increase the
198 preconcentration capacity (and the loading volume), we investigated an alternative grafting
199 approach on silica based monolith. Herein, a photografting process based on the thiol-ene
200 photoclick reaction is proposed to prepare monolayer-like functionalized silica monolithic
201 columns [12,16]. In first instance, we tried the “ene-thiol” version (referred as vinyl
202 functionalized silica and thiol-modified PBA in solution) as described by Chen et al [17]. But
203 we failed to produce stable PBA material. The grafts were leached-out during experiment
204 revealing adsorption rather than covalent grafting of the PBA derivatives on the support (data
205 not shown). The alternative “thiol-ene” version was then implemented on thiol-terminated
206 silica surface using vinyl-functionalized PBA derivative (AAPBA). After optimization of the
207 overall process (silane derivatization and photoclick grafting reactions) this chemical pathway
208 gives rise to silica monolith having $0.93 \text{ nmol cm}^{-1}$ (i.e $21 \text{ } \mu\text{mol } \mu\text{L}^{-1}$) of active sites, which
209 is 2 fold higher than our previous AAPBA functionalized monolith [4]. It was shown that high
210 concentration of reagents (up to 1M) is useful to prepare dense layers of grafted molecules
211 [16] but AAPBA monomer was not soluble up to 0.1 M. To increase the AAPBA grafting
212 density, it was intended to proceed to successive illumination steps (from 3 to 10), after
213 refreshing the solution inside the capillary in-between each illumination. The grafting density
214 increased up to $1.93 \pm 0.17 \text{ nmol cm}^{-1}$ for 3 successive grafting steps and then remained
215 constant.

216 This thiol-ene grafting pathway on silica monolith produces stable and dense phenylboronate
217 layers with a loss of active phenylboronate site inferior at 5% after ten affinity / elution
218 cycles.

219

220 3.2 Nucleosides affinity

221 Determination of affinity (K_d) for nucleosides were also determined using frontal affinity
222 breakthrough curves. Hydroquinone elutes at the column hold-time (not shown), whereas the
223 breakthrough curve plateaus are delayed for catechol (Figure S3). This result clearly indicates
224 that no non-specific interactions occur and that the retention of catechol is solely due to
225 boronate affinity. Table 1 gives the K_d values at pH 8.5 in a 15 mM phosphate buffer mobile
226 phase :

227 Table 1

228 The retention factor (k) for cis-diol compounds can be estimated through the K_d value
229 according to equation (2) [18]

$$230 \quad k = \frac{1}{K_d} \frac{B_{act}}{V_0} \quad (\text{Eq 3})$$

231 For example, a retention factor of about 18 is estimated for uridine with the experimental K_d
232 value of 2830 μM , a total number of active sites (B_{act}) of 2 nmol cm^{-1} and a hold-up volume
233 (V_0) of 40 nL for a 1-cm length monolithic column. The migration velocity of uridine in the
234 μBAMC (u') can be estimated by equation 3,

$$235 \quad u' = \frac{u}{1+k} \quad (\text{Eq 4})$$

236 where u is the mobile phase velocity. In other words, the maximum loading volume before
237 elution of a fraction of uridine should be about 18 times the column hold-up volume.

238

239

240 3.3 Multimodal chromatography: in-line coupling $\mu\text{BAMC-RP nano-LC}$

241 A capillary composed of a 1-cm length μBAMC unit coupled to a 7-cm length RP column
242 was used to evaluate the effectiveness of such in-line coupling strategy.

243 3.3.1 Determination of the loading capacity

244 The loading capacity of the 1-cm length photoclicked AAPBA monolith (μBAMC) was
245 evaluated by injecting small volumes of increasing uridine concentration (1 – 100 $\mu\text{g mL}^{-1}$).
246 Uridine was chosen as model solute due to its lowest affinity on the AAPBA silica monolith.

247

Figure 1

248 The peak area (Figure 1A) increases linearly with the concentration of uridine in the sample
249 solution up to approximately $50 \mu\text{g mL}^{-1}$ afterwards it can be noticed a deviation from the
250 linearity for the last point at $100 \mu\text{g mL}^{-1}$. According to figure 1, up to $50 \mu\text{g mL}^{-1}$ the
251 recovery is complete (the peak area increases proportionally to the amount injected). The
252 amount injected for a $50 \mu\text{g mL}^{-1}$ concentration and a 50 nL injection volume is 2.5 ng. Since
253 the length of the μBAMC is 1cm, the loading capacity is 2.5 ng cm^{-1} .

254 This capacity increases for nucleosides exhibiting higher boronate affinity. However,
255 regarding in-line coupling the preconcentration factor is a critical parameter. The maximum
256 loading volume was thus also investigated.

257 *3.3.2 Determination of the maximum loading volume and LOD for* 258 *uridine:*

259 As previously, after preconditioning, various injection volumes (from 50 to 1500 nL) of
260 uridine solution ($1 \mu\text{g mL}^{-1}$ in phosphate buffer pH 8.5) were loaded on the multimodal
261 column. After washing the μBAMC unit with a phosphate buffer pH 8.5, the peak area of
262 uridine was determined during the elution step. The plot of the recovered peak area versus
263 loading volume is presented Figure 1B. Experimentally, the breakthrough of uridine during
264 the loading step is observed after percolation of a sample volume of approximately 21
265 μBAMC hold-up volumes that is in good agreement with the previously estimated value (i.e
266 18 V_m). In order to demonstrate the usefulness of the presented in-line coupling to
267 preconcentrate cis-diol compounds, low concentration of uridine solutions solubilized in
268 phosphate buffer (pH 8.5) and in acetic acid (50 mM) were analyzed on the multimodal
269 column (Figure 2 (A)). Injection of uridine solubilized in phosphate buffer at pH 8.5 gives a
270 peak at 5.5 min (with a large S/N ratio) while no peak is detected when uridine is solubilized
271 in acetic acid (50 mM). The μBAMC unit is “activated” when the sample is loaded at pH 8.5.
272 Under acidic medium (i.e 50 mM acetic acid) the boronate affinity is turned off and no
273 preconcentration effect was observed (Figure 2A).

274 The quantity of uridine loaded (2.1 ng cm^{-1}) was lower than the maximum loading capacity
275 estimated previously. A loading volume of 1 μL (corresponding to 21 μBAMC hold-up
276 volume) was thus fixed as the maximum volume of sample that can be loaded on the 1-cm
277 length μBAMC unit of the multimodal column. Estimation of the limit of detection for uridine
278 was done by measuring the peak height in function of the concentration of uridine using a

279 loading volume of 1 μ L. LODs (S/N= 3) were estimated at 57 ng mL⁻¹ for uridine in such
280 experimental set-up. In addition, the linearity of the overall method is demonstrated by
281 plotting the corrected peak areas versus the analyte concentration Figure 2 (B).

282 Figure 2

284 3.4 Separation of nucleosides using multimodal chromatography (μ BAMC-RP nano-LC)

285 A proof of feasibility of this novel in-line coupling strategy was done for the preconcentration
286 and separation of 4 nucleosides (uridine, cytidine, adenosine and guanosine). 1 μ L of sample
287 (at a nucleoside concentration of 0.5 μ g mL⁻¹) was loaded on a preconditioned multimodal
288 column. The μ BAMC unit was washed with the same phosphate buffer (pH 8.5) before
289 elution and separation of nucleosides using acetic acid mobile phase. The elution and
290 separation of the first two peaks were done by continuously flushing the column with acetic
291 acid (50 mM). The mobile phase was then changed to acetic acid (50 mM)/MeOH (98/2, v/v)
292 (Figure 3). The 4 nucleosides were preconcentrated during the loading step. Elution is strong
293 enough to elute the captured nucleosides in a short injection plug in order to avoid detrimental
294 band broadening effect. This is of paramount importance to successful in-line coupling.
295 Indeed, performances of all in-line coupling strategies rely on the specificity to preconcentrate
296 efficiently and to transfer the captured target molecules to the separation area in the lowest
297 “injection” volume. The base-line separation of the four nucleosides on the reversed-phase
298 monolithic column demonstrates the power of the presented in-line coupling.

299

300 4- Conclusion :

301 The elaboration of miniaturized boronate affinity monolithic column through thiol-ene
302 photoclick chemistry was shown to greatly improve the surface density of active sites on
303 silica monolith. The thiol-ene version of such photo-initiated reactions allows to produce
304 stable and dense layer-like phenylboronate surface of 1.93 ± 0.17 nmol cm⁻¹ for 3 successive
305 grafting steps. With regards to the previously photopolymerization pathway, this method
306 allows to increase the surface density by a factor 4. High binding capacity is a prerequisite to
307 in-line coupling of such μ BAMC for preconcentration and purification purpose. We evaluated

308 the retention behavior of such AAPBA photoclicked columns with nucleosides and
309 determined the optimal capture conditions. The coupling with a reversed-phase capillary
310 column was then investigated. Determination of the capacity and loading volume on a
311 composite capillary composed of 1-cm length μ BAMC unit with 7-cm length reversed-phase
312 monolithic column demonstrated the potentiality of such coupling at the capillary scale. The
313 evaluation of this novel in-line coupling was done on a capillary electrophoresis system under
314 pressurization mode. NanoLC experiments with such experimental set-up allows to control
315 the injection volume and to use a large diversity of mobile phases owing to absence of pump
316 and injection valve. Using the least retained nucleoside (uridine) it was shown that a loading
317 volume as large as 20 μ BAMC hold-up volume can be loaded without breakthrough of
318 sample from the preconcentration unit. This preconcentration factor allows to greatly decrease
319 the nucleosides concentration that can be detected. The in-line coupling was successfully
320 applied to the preconcentration and separation of 4 nucleosides.

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387

388 **Figure captions**

389 Figure 1. Plots of the peak area in function of the concentration of uridine at a constant
390 loading volume of 50 nl (1.2 μ BAMC volume) (A) and for increasing loading volume at a
391 constant uridine concentration of 1 μ g mL⁻¹ (B). The multimodal capillary column is
392 composed of a 1-cm length μ BAMC unit connected to a 7-cm length reversed-phase
393 monolithic column. The percolation volume was increased from 50 to 1420 nL and uridines
394 standard solutions at 1, 5, 20, 50 and 100 μ g mL⁻¹ in 15mM phosphate (pH 8.5) were
395 percolated at 0.01 cm min⁻¹.

396 Figure 2. Capture and elution of uridines solubilized in phosphate buffer pH 8.5 (black curve)
397 and in acetic acid (grey curve) on a μ BMAC-RP multimodal capillary column (A) and plot of
398 the evolution of the peak area versus concentration of uridine in binding buffer (B). Length of
399 the μ BAMC segment: 1 cm; length of the reversed-phase segment: 7 cm; mobile phase 50
400 mM acetic acid. The multimodal capillary was preconditioned with binding buffer (15 mM
401 phosphate buffer at pH 8.5) prior to injection of 1 μ l (corresponding to 21 volumes of the
402 μ BAMC unit) of uridine. Before elution and separation, the μ BAMC unit was washed with 50
403 nl of binding buffer.

404 Figure 3. Preconcentration and separation of the 4 nucleosides on multimodal capillary. The
405 multimodal capillary (1-cm length μ BAMC – 7 cm-length RP-capillary column) was
406 preconditioned with binding buffer (15 mM phosphate buffer at pH 8.5) prior to injection of 1
407 μ l (corresponding to 21 volumes of the μ BAMC unit) of the 4 nucleosides at a concentration
408 of 0.5 μ g mL⁻¹ each. Before elution and separation, the μ BAMC unit was washed with 50 nl
409 of binding buffer. Elution and separation of the 4 nucleosides was done using an acetic
410 acid/MeOH (92/8, v/v) mobile phase.