1 Simultaneous analysis for water- and fat-soluble vitamins by a novel single
2 chromatography technique unifying supercritical fluid chromatography and liquid
3 chromatography

4
5 Kaori Taguchi¹, Eiichiro Fukusaki¹, Takeshi Bamba^{1*}

6 *Department of Biotechnology, Graduate School of Engineering, Osaka University,*

7 *2-1 Yamadaoka, Suita, Osaka 565-0871, Japan¹*

8

9 * Corresponding author at: Department of Biotechnology, Graduate School of
10 Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.

11 phone/fax: +81-(0)6-6879-7418.

12 *E-mail address:* bamba@bio.eng.osaka-u.ac.jp (T. Bamba).

13 **Abstract**

14 Chromatography techniques usually use a single state in the mobile phase, such as
15 liquid, gas, or supercritical fluid. Chromatographers manage one of these techniques for
16 their purpose but are sometimes required to use multiple methods, or even worse, multiple
17 techniques when the target compounds have a wide range of chemical properties. To
18 overcome this challenge, we developed a single method covering a diverse compound
19 range by means of a “unified” chromatography which completely bridges supercritical
20 fluid chromatography and liquid chromatography. In our method, the phase state was
21 continuously changed in the following order; supercritical, subcritical and liquid.
22 Moreover, the gradient of the mobile phase starting at almost 100% CO₂ was replaced
23 with 100% methanol at the end completely. As a result, this approach achieved further
24 extension of the polarity range of the mobile phase in a single run, and successfully
25 enabled the simultaneous analysis of fat- and water-soluble vitamins with a wide log *P*
26 range of -2.11 to 10.12. Furthermore, the 17 vitamins were exceptionally separated in 4
27 min. Our results indicated that the use of dense CO₂ and the replacement of CO₂ by
28 methanol are practical approaches in unified chromatography covering diverse
29 compounds. Additionally, this is a first report to apply the novel approach to unified
30 chromatography, and can open another door for diverse compound analysis in a single

31 chromatographic technique with single injection, single column and single system.

32

33 [**Key words:** unified chromatography; supercritical fluid chromatography; mass

34 spectrometry; fat soluble vitamins; water soluble vitamins]

35 **1. Introduction**

36 Supercritical fluid chromatography (SFC) is a complementary separation technique
37 to gas chromatography (GC) and liquid chromatography (LC). This technique employs
38 supercritical carbon dioxide or subcritical carbon dioxide as the mobile phase. Such fluids,
39 which is near or above its critical temperature and/or pressure of CO₂, is endowed with
40 low viscosity and high diffusivity resulting in faster and better separation. Supercritical
41 fluid CO₂, the main fluid of the mobile phase in SFC, is non-polar and similar to *n*-hexane
42 [1], thus this technique was considered as normal phase chromatography and a suitable
43 technique for non-polar compounds. However, the very non-polar fluid is miscible with
44 polar organic solvents like methanol and ethanol that are used as modifier. Additionally,
45 it accepts acid, base and even water thus this characteristic enables further extension of
46 the polarity range of the mobile phase. Consequently, SFC gains the capability to cover a
47 wide polarity range of separation [2] and is therefore applicable to the analysis of both
48 polar and non-polar compounds, which are opposite attributes. We think that SFC has
49 great potential for the analysis of diverse compounds and could allow us to shift into a
50 single method as well as single chromatographic technique which typically require two
51 separated methods. However only a very few reports utilized SFC for simultaneous
52 analysis of polar and non-polar compounds [3,4]. Therefore further investigation is

53 necessary in order to get some insights in the applicability of SFC to diverse compound
54 analysis.

55 Vitamins are essential dietary nutrients for animals and humans that are required
56 for the normal growth, self-maintenance and functioning of human and animal bodies.
57 Additionally, the lack or excess of vitamins in the organism's system produces specific
58 diseases [5]. Therefore the determination of vitamins in food or bodies has been studied
59 extensively [6–14]. Vitamins are classified into two groups according to their solubility
60 namely, fat-soluble-vitamin (FSV) and water-soluble-vitamin (WSV) [15]. There is a
61 huge difference between the two groups in water solubility, in which the log *P* range is
62 from -2.11 to 10.12 as shown in Table 1. Moreover, it is difficult to analyze FSV and
63 WSV simultaneously because their chemical characteristics including their acid-base
64 behavior are also different [16]. Therefore, we considered that the simultaneous analysis
65 of vitamins could be a good practical and indicative probe to verify the ability of SFC to
66 extend the compound coverage in a single run.

67 The aim of this study was to figure out the applicable scope of SFC. FSV and WSV
68 were used in order to get some insights in simultaneous diverse compound analysis using
69 SFC. In this paper, we developed a method analyzing FSV and WSV which cover various
70 chemical properties and a wide range of polarities, and then discussed its usability using

71 the result.

72

73 **2. Materials and Methods**

74 *2.1. Chemicals*

75 Methanol, acetonitrile and 2-propanol (optima LC/MS) were purchased from
76 Fisher Scientific (Pittsburg, PA). Chloroform, Formic acids (88%, certified ACS),
77 ammonium formate (LC/MS), ammonium hydroxide, retinol acetate (A acetate), retinol
78 palmitate (A palmitate), *trans*- β -carotene (β -carotene), ergocalciferol (D2), *dl*- α -
79 tocopherol (α -tocopherol), *dl*- α -tocopherol acetate (α -tocopherol acetate), K1, K2,
80 thiamine hydrochloride (thiamine), riboflavin, nicotinic acid, nicotinamide, D-
81 pantothenic acid hemicalcium (D-pantothenic acid), pyridoxine, biotin, cyanocobalamin
82 (B12), and *l*-ascorbic acid (VC) were purchased from Sigma-Aldrich (St. Louis, MO).
83 Carbon dioxide (medical grade), which was used as the mobile phase in SFC, was
84 purchased from Airgas, Inc. (Radnor, PA). De-ionized water was prepared by a Milli-Q
85 system (Millipore, MA).

86

87 *2.2. SFC/MS/MS Analysis*

88 SFC/MS/MS analysis was performed using an ACQUITY UltraPerformance

89 Convergence Chromatography (UPC²) system (Waters, Milford, MA) with a Xevo TQD
90 tandem mass spectrometer (Waters). The SFC system was equipped with a convergence
91 manager which controls backpressure, binary solvent manager, temperature-controlled
92 column manager and fixed loop sample manager. The MS was equipped with an ESI
93 source. The flow was split to MS and convergence manager after a column so that a single
94 phase CO₂/methanol mixture can be maintained. The SFC conditions for column
95 screening were as follows: mobile phase, SCCO₂ with the following modifiers; 2-
96 propanol, acetonitrile, methanol, methanol/water (95/5, v/v) with 0.1% (v/v) formic acid;
97 injection volume, 1 µL; flow rate, 1.0 mL/min; modifier gradient; 2% (0.5min), 2–60%
98 (4.5 min), 60% (3.5 min), 60–2% (0.5 min), 2% (1.0 min); temperature of column
99 manager, 40°C; temperature of sample manager, 6°C; active back pressure regulator
100 (ABPR), 12.4 MPa; columns, ACQUITY UPC² BEH 2-Ethylpyridine (2-EP), ACQUITY
101 UPC² CSH Fluoro-Phenyl (FP), ACQUITY UPC² HSS C18 SB (C18 SB), ACQUITY
102 HSS Cyano (Cyano), ACQUITY CSH Phenyl-Hexyl (PH) (Waters, 100 × 3.0 mm i.d.,
103 particle size: sub 2 µm). The final MS analysis conditions were as follows: capillary
104 voltage, 0.8 kV; desolvation temperature, 600°C; cone gas flow rate, 50L/h; desolvation
105 gas flow rate, 800 L/h. The multiple reaction monitoring (MRM) was employed in this
106 study (Supplemental Table 1). The SFC/ESI-MS/MS system was controlled by MassLynx

107 version 4.1 (Waters). MassLynx version 4.1 equipped with application manager
108 TargetLynx was used for acquisition and processing of the SFC/ESI-MS/MS data.

109

110 *2.3. Sample Preparation*

111 Standard stock solutions were prepared by dissolving each compound in
112 appropriate diluents. The diluent was 50:50 chloroform:methanol for FSV, 50:50
113 methanol:water for WSV and 100% water for VC. The stock solutions were further
114 diluted with methanol to obtain the final mixed standard solutions.

115

116

117 **3. Results**

118 *3.1. Investigation of column and modifier*

119 We executed column screening using BEH, 2-EP, cyano, FP, C18SB and PH
120 columns with 2-propanol, acetonitrile, methanol, and methanol/water (v/v 95/5). All
121 modifiers contained 0.1% (v/v) formic acid to help ionization. According to the peak
122 shape and the number of detected peaks (Table 2), we narrowed down the candidates to
123 three. Specifically, C18SB using methanol, C18SB using methanol/water (v/v 95/5), and
124 FP using methanol eluted most of compounds included in our column screening mixture.

125 FP column uses propyl fluorophenyl ligands and eluted all vitamins. However, FSV was
126 less retentive with narrower retention window in the FP column compared to the C18SB.
127 The C18SB column uses an octadecyl carbon chain bonded silica and possesses the most
128 hydrophobic interaction with non-polar compounds among screening columns. We
129 assumed that this is the reason why the C18SB column retained FSV very well. On the
130 other hand, the C18SB column can retain hydrophilic compounds due to hydrophilic
131 interaction by residual silanol groups on non-encapping stationary phase. We thought
132 that this oppositional characteristics of C18 and silanol provided important retention
133 behavior to both FSV and WSV; the octadecyl carbon chain helped to retain FSV and
134 silanol groups on base particles assisted to retain WSV. As a result, even a single column
135 was able to cover compounds with a wide range of polarities. There was no significant
136 difference between methanol and methanol/water as a modifier according to the number
137 of detected peaks, while the observed peak shape in methanol was slightly better than
138 methanol/water in C18SB. Based on these observations, we decided to use the C18SB
139 column with methanol for further experiments.

140

141 *3.2. Investigation of additives*

142 Although the C18SB column with methanol modifier retained both FSV and WSV,

143 there were a couple of issues in the chromatogram. One was the missing peak of thiamine
144 and the other was the peak shapes of pyridoxine and VC. Because of over-retention,
145 thiamine was not eluted from the C18SB column. Although thiamine was observed with
146 longer acquisition time, it was still over retained compared to other vitamins. On the other
147 hand the peak tailing was observed in pyridoxine and VC. Pyridoxine contains a
148 heterocyclic amine ring, so we assumed that the compound has interaction with silanol
149 groups in the column. Berger showed peak shape improvement of base compounds by the
150 addition of a basic additive to the mobile phase [17]. Also, Perrenoud et al. reported that
151 the presence of ammonium hydroxide in mobile phase in SFC provided improvement of
152 the peak shape on basic analytes [18]. Therefore the influence of bases as additives on the
153 peak tailing of pyridoxine was examined. As shown in Fig. 1, the peak shape of
154 pyridoxine was completely improved by using ammonium hydroxide, but it was not
155 changed by the addition of formic acid. In addition to this, we confirmed that the same
156 improvement was observed in pyridoxine with the use of ammonium formate as an
157 additive (Supplemental Fig. 1). Based on the results, it was supposable that ammonium
158 ion disrupted the interactions between silanol residues and pyridoxine with amine on the
159 C18SB column resulting in the improvement of the peak shape of pyridoxine.

160 The peak tailing of pyridoxine was improved but the issues regarding thiamine

161 (over retention) and VC (peak tailing) still remained. Before further investigation of
162 additives, we took into account the use of a shorter column; allowing the use of decreasing
163 silanol groups which might result in the improvement of the aforementioned issues. Fig.
164 2 shows the results of 5cm and 10cm column. Thiamine was eluted within 7min with
165 better peak shape in the 5cm column, and pyridoxine also showed better peak shape.
166 Unfortunately, no change was observed in the peak shape of VC.

167 Brunelli et al. [19] suggested the use of basic as well as acid additives together with
168 modifier to improve peak shapes if samples contain basic and acidic compounds. Berger
169 [17, 20] also figured out that the effective additives for acidic solutes for improvement of
170 the peak shape were stronger acids in general and suppression of solute ionization by
171 acidic additives was a very important factor for peak tailing of carboxylic acids in SFC.
172 Although the use of strong acid can improve the peak shape of VC, it was hard for us to
173 use a strong acid such as trifluoroacetic acid causing background noise on MS configured
174 in this system. Therefore, we investigated if we can achieve a similar improvement in VC
175 using a weak acid specifically formic acid. As shown in Fig. 3, the increase in formic acid
176 concentration brought a positive effect and improved the peak shape of VC. While, it
177 distorted the peak shape of pyridoxine. Also the sensitivity was dropped in B12, which
178 ion suppression is caused by the addition of formic acid [21]. However, the peak width

179 was improved in VC by increasing the ammonium formate concentration without any
180 negative effects on other peaks (Fig. 4).

181 The result implied that the concentration of additives was another key factor to
182 improve the peak shape of VC in the separation. Water was also added as additives in
183 order to make it easy to prepare the modifier by dissolving ammonium formate into water,
184 but the modification did not have any negative effect in the separation. In fact, it helped
185 to improve the peak shape of B12.

186

187 *3.3. Extension of Modifier Composition*

188 Peak shapes in this separation were improved after consideration of the additives.
189 However, the peak shapes were still not acceptable especially in VC and B12. As a rule
190 of thumb, a preferred analyte in SFC is soluble at least 1mg/mL in methanol [22]. Actually
191 VC was not an amenable compound to SFC since the solubility was lower than that
192 criteria in methanol. As for B12, it was dissolved in methanol at 1mg/mL but it contains
193 a phosphate group which brings a hydrophilic characteristic into a compound. So we
194 assumed that solvating power of the mobile phase is not strong enough to elute VC and
195 B12. Based on this assumption, the increase of polarity in mobile phase was investigated
196 for elution of the compounds with better peak shape.

197 The easiest way to make the modifier polar is by the addition of water. However,
198 5% water was already added and addition of more water sometimes brings unstable
199 system pressure seemly caused by a miscibility issue. Therefore we took another path by
200 increasing the modifier composition in the gradient up to 100%. When the final
201 composition of modifier in the gradient changes from 70 to 100%, the peak width was
202 dramatically improved from 0.23 min to 0.11 min, and 0.25 min to 0.10min in VC and
203 B12, respectively. As a result, 17 vitamins were separated in 4 min as shown in Fig. 5,
204 and the separation time was faster than any of other reported method [5,16,23–25]. Also
205 we confirmed the reproducibility of each vitamin. The method showed good
206 reproducibility of retention time ranging from 0 to 0.51%, otherwise the area
207 reproducibility ranged from 1.24 to 15.63% (Table 3).

208

209

210 **4. Discussion**

211 Chromatography techniques are usually classified according to the physical state
212 of the mobile phase such as gas, supercritical fluid or liquid. Therefore, it might cause
213 confusion if we classify our method as supercritical fluid chromatography since the phase
214 state observed in the chromatography is not only supercritical. The critical parameters, to

215 keep CO₂ as supercritical, continuously changes along with a change in the modifier
216 composition. Unless the operating temperature and pressure are set above the critical
217 points which are updated on a moment-to-moment basis in a gradient method, the phase
218 state of CO₂ may no longer be supercritical at some points [26]. So our developed method
219 utilizes three states namely supercritical, subcritical and liquid. This kind of separation
220 technique using more than one physical state of mobile phase can be classified as unified
221 chromatography [26–28]. The very important key in unified chromatography is, no
222 discontinuous transition is observed in the mobile phase even if CO₂ changes the phase
223 state [26]. Therefore the mobile phase can be changed from gas to supercritical, or
224 supercritical to liquid consecutively by controlling temperature or pressure, and CO₂
225 always exists in unified chromatography throughout separation.

226 When we intend to control and change the phase state by increasing the
227 composition of modifier, it is crucial to consider the phase diagram of the mobile phase.
228 Although the binary mobile phase mixing CO₂ and modifier can lead to gas, supercritical
229 and liquid state by changing the composition of mobile phase, it also leads to co-existing
230 of gas and liquid phases under certain conditions. The formation of two phases can be
231 avoided by working at a high pressure, high temperature, and the parameters of column
232 temperature at 40°C and ABPR pressure above 10.3_MPa are enough high to keep away

233 from the two phase-region over full composition range of modifier [28]. In our method,
234 the phase state of CO₂ was supercritical at the start, then it was presumably changed to
235 subcritical and liquid by increasing the modifier composition. However, there is more to
236 this method. CO₂ is completely replaced with methanol, so this approach can enhance the
237 solvating power range of mobile phase compared to SFC, LC, and even conventional
238 unified chromatography. In other words, the approach can provide the possibility of
239 analyzing diverse compounds with a wide polarity range. Additionally, it blurs the
240 distinction between SFC and LC, and the two chromatographic techniques are completely
241 “unified”. However, there is another essential key to achieve the unified chromatography;
242 suitable column selection increases the possibility for simultaneous analysis of diverse
243 compounds. We selected a C18SB column with C18 ligand bonded to non-encapped
244 silica phase. In terms of column bed polarity, it is hydrophobic at the ligand, whereas
245 hydrophilic at silanol residues. The opposed retention behavior at different sites in the
246 column, led to the retention diversity from hydrophobic to hydrophilic compounds in the
247 separation.

248

249

250 **5. Conclusion**

251 The results indicate an overture for simultaneous analysis for diverse compounds
252 using unified chromatography which completely bridges SFC and LC. Our goal in this
253 study was to figure out the usability of SFC for simultaneous analysis of compounds with
254 diverse polarities (in this case, vitamins). From the perspective of the original goal, the
255 solvating power of mobile phase was limited in SFC, it was not fully-usable for the
256 analysis if we had not gone far beyond SFC. Beyond SFC, there is unified
257 chromatography which enables to widen the diversity of polarity in the mobile phase.

258 Unified chromatography is an extended technique of SFC because it is not
259 achievable without dense CO₂. Our novel approach, which uses dense CO₂ and its
260 replacement by methanol, enhanced the diversity of the mobile phase polarity. This is a
261 universal approach to unified chromatography. Consequently 17 vitamins with diverse
262 chemical properties separated in 4min. The results showed that unified chromatography
263 is practical and is ready for application. Specifically, the novel approach to unified
264 chromatography is useful and can offer an alternative separation for diverse compounds
265 with a wide range of polarities. In the near future, unified chromatography with our
266 approach opens a lot of doors and is possible to be the first choice of separation technique
267 for chromatographers.

268

269

270 **6. Acknowledgements**

271 This work was partially supported by the Development of Systems and Technology
272 for Advanced Measurement and Analysis Project (JST).

273

274

275 **References**

- 276 [1] T. Bamba, N. Shimonishi, A. Matsubara, K. Hirata, Y. Nakazawa, A. Kobayashi,
277 E. Fukusaki, High throughput and exhaustive analysis of diverse lipids by using
278 supercritical fluid chromatography-mass spectrometry for metabolomics, J.
279 Biosci. Bioeng. 105 (2008) 460-469.
- 280 [2] L.T. Taylor, Packed column supercritical fluid chromatography of hydrophilic
281 analytes via water-rich modifiers, J. Chromatogr. A 1250 (2012) 196-204.
- 282 [3] M. Ishibashi, T. Ando, M. Sakai, A. Matsubara, T. Uchikata, E. Fukusaki, T.
283 Bamba, High-throughput simultaneous analysis of pesticides by supercritical
284 fluid chromatography/tandem mass spectrometry, J. Chromatogr. A 1266 (2012)
285 143-148.
- 286 [4] K. Taguchi, E. Fukusaki, T. Bamba, Simultaneous and rapid analysis of bile acids
287 including conjugates by supercritical fluid chromatography coupled to tandem
288 mass spectrometry, J. Chromatogr. A 1299 (2013) 103-109.
- 289 [5] P. Moreno, V. Salvadó, Determination of eight water- and fat-soluble vitamins in
290 multi-vitamin pharmaceutical formulations by high-performance liquid
291 chromatography, J. Chromatogr. A 870 (2000) 207-215.
- 292 [6] R. Mittermayr, A. Kalman, M.J. Trisconi, O. Heudi, Determination of Vitamin

293 Bs in a range of fortified food products by reversed-phase liquid
 294 chromatography–mass spectrometry with electrospray ionisation, J. Chromatogr.
 295 A 1032 (2004) 1-6

296 [7] M.A. Kall, Determination of total vitamin B₆ in foods by isocratic HPLC: a
 297 comparison with microbiological analysis, Food Chem. 82 (2003) 315-327.

298 [8] O. Heudi, T. Kiliç, P. Fontannaz, E. Marley, Determination of Vitamin B₁₂ in
 299 food products and in premixes by reversed-phase high performance liquid
 300 chromatography and immunoaffinity extraction, J. Chromatogr. A 1101 (2006)
 301 63-68.

302 [9] H. Wakabayashi, K. Onodera, S. Yamato, K. Shimada, Simultaneous
 303 determination of vitamin K analogs in human serum by sensitive and selective
 304 high-performance liquid chromatography with electrochemical detection,
 305 Nutrition 19 (2003) 661-665.

306 [10] K. Kuwahara, A. Nanri, N. M. Pham, K. Kurotani, A. Kume, M. Sato, K. Kawai,
 307 H. Kasai, T. Mizoue, Serum vitamin B₆, folate, and homocysteine concentrations
 308 and oxidative DNA damage in Japanese men and women, Nutrition 29 (2013)
 309 1219-1223.

310 [11] T. Fukuwatari, E. Sugimoto, T. Tsuji, J. Hirose, T. Fukui, K. Shibata, Urinary

311 excretion of vitamin B12 depends on urine volume in Japanese female university
312 students and elderly, *Nutr. Res.* 29 (2009) 839-845.

313 [12] E.M. Malone, G. Dowling, C.T. Elliott, D.G. Kennedy, L. Regan, Development
314 of a rapid, multi-class method for the confirmatory analysis of anti-inflammatory
315 drugs in bovine milk using liquid chromatography tandem mass spectrometry, *J.*
316 *Chromatogr. A* 1216 (2009) 8132-8140.

317 [13] T. Plozza, V. Craige Trenerry, D. Caridi, The simultaneous determination of
318 vitamins A, E and β -carotene in bovine milk by high performance liquid
319 chromatography–ion trap mass spectrometry (HPLC–MSⁿ), *Food Chem.* 134
320 (2012) 559-563.

321 [14] O. Guneser, Y. Karagul, Y.K. Yuceer, Effect of ultraviolet light on water- and fat-
322 soluble vitamins in cow and goat milk, *J. Dairy Sci.* 2012, 95, 6230–6241.

323 [15] D. Nohr, Vitamins, in: J.W. Fuquay, P.F. Fox, P.L.H. McSweeney (Eds.),
324 *Encyclopedia of Dairy Sciences*, Second Ed, Academic Press, Amsterdam, 2011,
325 pp636–638.

326 [16] M. Delgado-Amarreño, I. González-Maza, A. Sánchez-Pérez, R. Carabias-
327 Martinez, Separation and simultaneous determination of water-soluble and fat-
328 soluble vitamins by electrokinetic capillary chromatography, *J. Chromatogr. A*

329 953 (2002) 257-262.

330 [17] T. A. Berger, Separation of polar solutes by packed column supercritical fluid
 331 chromatography, J. Chromatogr. A 785 (1997) 3-33.

332 [18] A. Grand-Guillaume Perrenoud, J. Boccard, J.-L. Veuthey, D. Guillarme,
 333 Analysis of basic compounds by supercritical fluid chromatography: attempts to
 334 improve peak shape and maintain mass spectrometry compatibility, J.
 335 Chromatogr. A 1262 (2012) 205-213.

336 [19] C. Brunelli, Y. Zhao, M.-H. Brown, P. Sandra, Development of a supercritical
 337 fluid chromatography high-resolution separation method suitable for
 338 pharmaceuticals using cyanopropyl silica, J. Chromatogr. A 1185 (2008) 263-
 339 272.

340 [20] T.A. Berger, J.F. Deye, Role of additives in packed column supercritical fluid
 341 chromatography: suppression of solute ionization, J. Chromatogr. 547 (1991)
 342 377-392.

343 [21] X. Luo, B. Chen, L. Ding, F. Tang, S. Yao, HPLC-ESI-MS analysis of Vitamin
 344 B₁₂ in food products and in multivitamins-multimineral tablets, Anal. Chim.
 345 Acta 562 (2006) 185-189.

346 [22] C. West, How good is SFC for polar analytes?, Chromatogr. Today May/June

347 (2013) 22-27.

348 [23] B. Klejdus, J. Petrlová, D. Potěšil, V. Adam, R. Mikelová, J. Vacek, R. Kizek, V.

349 Kubáň, Simultaneous determination of water- and fat-soluble vitamins in

350 pharmaceutical preparations by high-performance liquid chromatography

351 coupled with diode array detection, *Anal. Chim. Acta* 520 (2004) 57-67.

352 [24] R. Dabre, N. Azad, S. Schwämmle, M. Lämmerhofer, W. Lindner, Simultaneous

353 separation and analysis of water- and fat-soluble vitamins on multi-modal

354 reversed-phase weak anion exchange material by HPLC-UV, *J. Sep. Sci.* 34

355 (2011) 761-772.

356 [25] C. Yin, Y. Cao, S. Ding, Y. Wang, Rapid determination of water- and fat-soluble

357 vitamins with microemulsion electrokinetic chromatography, *J. Chromatogr. A*

358 1193 (2008) 172-177.

359 [26] T.L. Chester, J.D. Pinkston, Supercritical fluid and unified chromatography, *Anal.*

360 *Chem.* 76 (2004) 4606-4613.

361 [27] D. Tong, K. D. Bartle, A.A. Clifford, Principles and applications of unified

362 chromatography, *J. Chromatogr. A* 703 (1995) 17-35.

363 [28] P.S. Wells, S. Zhou, J.F. Parcher, Unified chromatography with CO₂-based

364 binary mobile phases, *Anal. Chem.* 75 (2003) 18A-24A.

366 **Figure legends**

367 **Fig. 1.** MRM chromatograms of 14 vitamins using methanol with A: 0.1% (v/v) formic
368 acid, B: 0.2% (v/v) formic acid, and C: 0.1% (v/v) formic acid and 0.1% (v/v) ammonium
369 hydroxide. 1: A acetate and A palmitate, 2: α -Tocopherol, 3: K2, 4:K1, 5: β -Carotene, 6:
370 Nicotinamide, 7: Nicotinic acid, 8: Pyridoxine, 9: D-Pantothenic acid, 10: Biotin, 11:
371 Thiamine, 12: Riboflavin, 13: VC.

372

373 **Fig. 2.** MRM chromatograms of 13 vitamins with different column length of a C18SB
374 column. A: 5cm column, B: 10cm column. 1: A acetate and A palmitate, 2: α -Tocopherol,
375 3: K2, 4:K1, 5: β -Carotene, 6: Nicotinamide, 7: Nicotinic acid, 8: Pyridoxine, 9: D-
376 Pantothenic acid, 10: Biotin, 11: Thiamine, 12: VC

377

378 **Fig. 3.** The effect of formic acid in different concentrations. MRM chromatograms of A:
379 B12, B: VC, C: Pyridoxine, D: Thiamine using 1: 0.1% (w/v) ammonium formate without
380 formic acid, 2: with 0.2%, 3: with 0.4%, 4: with 0.6% (v/v) formic acid in methanol.

381

382 **Fig. 4.** The effect of ammonium formate concentration on peak shapes. MRM
383 chromatograms of 16 vitamins using A: 0.2% (w/v) ammonium formate in

384 methanol/water (95/5 v/v), B: 0.1% (w/v) ammonium formate in methanol/water (95/5
385 v/v). 1: A acetate, 2: A palmitate, 3: α -Tocopherol, 4: K2, 5: K1, 6: β -Carotene, 7:
386 Nicotinamide, 8: Nicotinic acid, 9: Pyridoxine, 10: D-Pantothenic acid, 11: Biotin, 12:
387 Thiamine, 13: Riboflavin, 14: VC, 15: B12.

388

389 **Fig. 5.** MRM chromatograms of 17 vitamins. 1: A acetate, 2: A palmitate, 3: D2, 4: α -
390 Tocopherol, 5: K2, 6: K1, 7: α -Tocopherol acetate, 8: β -Carotene, 9: Nicotinamide, 10:
391 Nicotinic acid, 11: Pyridoxine, 12: D-Pantothenic acid, 13: Biotin, 14: Thiamine, 15:
392 Riboflavin, 16: B12, 17: VC. Method conditions as follows; modifier: methanol/water
393 (95/5, v/v) with 0.2% ammonium formate; gradient condition: 2% (0.5 min), 2–30% (2.0
394 min), 30–85% (0.8 min), 85% (2.7min), 85–100% (0.2 min), 100% (1.3 min), 100–2% (1
395 min), 2% (1.5 min); flow rate: 1.2 mL/min at a column temperature of 40°C; back
396 pressure: 15.2 MPa (6.0 min), 15.2–10.3 MPa (0.2 min), 10.3 MPa (1.6min), 10.3–15.2
397 MPa (0.5min), 15.2 MPa (1.7min).

398

399

400

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1

Classification of vitamin

	Vitamin	Class	Log <i>P</i>
1	Retinol	FSV	6.38
2	Retinyl acetate (A acetate)	FSV	6.56
3	Retinyl palmitate (A palmitate)	FSV	10.12
4	α -tocopherol	FSV	7.59
5	Ergocalciferol (D2)	FSV	8.84
6	K1	FSV	8.48
7	K2	FSV	9.01
8	β -carotene	FSV	9.72
9	Tiamine	WSV	-2.11
10	Riboflavin	WSV	-1.05
11	Nicotinic acid	WSV	0.29
12	Niacinamide	WSV	-0.45
13	D-Pantothenic acid	WSV	-1.12
14	Pyridoxine	WSV	-0.57
15	Biotin	WSV	0.17
16	Cyanocobalamin (B12)	WSV	0.67
17	Ascorbic acid (VC)	WSV	-1.58

Table 2

Retention time in column screening

Column	Modifier	A palmitate (min)	A acetate (min)	α -tocopherol (min)	K2 (min)	K1 (min)	β -carotene (min)	Nicotinamide (min)	Nicotinic acid (min)	Pyridoxin (min)	D-Pantothenic acid (min)	Biotin (min)	Thiamine (min)	Riboflavin (min)	VC (min)
BEH	IPA	0.62	0.62	1.65	0.86	0.80	0.88	4.76	3.19	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	ACN	1.42	1.42	1.94 ^a	1.67	1.44	0.89	6.99	4.84	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	0.64	0.67	1.49 ^a	0.68	0.68	0.68	3.33	2.83	N.D.	3.29	3.82	N.D.	4.13 ^{ab}	N.D.
	95% MeOH	0.58	0.58	1.16	0.70	0.64	0.61	3.22	3.05 ^a	4.37 ^a	3.31	3.56	5.53 ^a	3.94 ^{ab}	N.D.
2-EP	IPA	0.88	1.12	2.84	1.50	1.02 ^a	2.16 ^a	4.07	4.02	4.61 ^{ab}	4.07	N.D.	N.D.	N.D.	N.D.
	ACN	1.13	1.13	2.64	1.81	1.37 ^a	2.00	5.27	8.81 ^{ab}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	0.67	0.87	2.37	1.33 ^a	1.00 ^a	1.82	3.00	3.24	N.D.	3.36	3.66	N.D.	N.D.	4.64 ^a
	95% MeOH	0.71	0.71	1.60 ^a	0.96	0.84	0.97	3.06	3.42	3.00	N.D.	3.56 ^b	4.26 ^a	N.D.	4.32 ^a
Cyano	IPA	0.81	1.32	1.63	1.24	1.11	2.60	4.08	3.19 ^a	N.D.	N.D.	N.D.	N.D.	N.D.	4.75 ^a
	ACN	0.89	1.50	1.24 ^a	1.48	1.27	2.13	5.73 ^a	4.63 ^a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	0.88	1.35	1.70	1.29	1.15	2.41	2.92	2.73 ^a	N.D.	2.81 ^a	3.28 ^a	N.D.	3.24 ^b	2.80 ^b
	95% MeOH	0.87	1.06	1.62 ^a	1.06	1.00	2.46	2.87	2.60	4.73 ^a	2.92 ^a	3.02	4.86 ^a	N.D.	2.95 ^b
FP	IPA	0.75	0.80	1.58 ^a	0.84	0.82	0.89	4.63	3.90 ^a	6.40 ^b	N.D.	N.D.	N.D.	N.D.	4.80 ^a
	ACN	1.10	2.00	1.18	1.15	1.08	1.10	6.20	7.76	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	0.73	0.73	1.60	0.91	0.88	0.92	3.04	2.91	3.83 ^a	2.92	3.31	5.32 ^a	3.60	3.62 ^a
	95% MeOH	0.69	0.69	1.29	0.75	0.74	0.75	3.01	2.99	3.54 ^a	3.04 ^b	3.11 ^b	4.71 ^a	N.D.	2.86 ^a
C18SB	IPA	1.40	1.40	3.00	2.29	2.28	3.39	5.75	3.25	3.60 ^b	4.80 ^b	N.D.	N.D.	N.D.	4.22 ^a
	ACN	2.17	3.13	N.D.	2.73	2.74	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	1.52	2.60	2.87	2.26	2.26	3.43	3.49	2.58	4.37 ^a	2.94	3.69	N.D.	4.01	3.16 ^a
	95% MeOH	1.20	2.50	2.85	2.19	2.18	3.24	2.99	2.64 ^a	3.56 ^a	2.73	2.98	6.97 ^a	N.D.	2.80 ^a
PH	IPA	0.86	1.68	1.70	1.47	1.36	2.83	2.84 ^a	2.64 ^a	3.70 ^a	N.D.	N.D.	N.D.	N.D.	N.D.
	ACN	0.93	1.75	1.86	1.56	1.46	2.58	3.00 ^a	3.96 ^a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	MeOH	0.90	1.81	1.80	1.58	1.45	2.88	1.52 ^a	2.00 ^a	3.16 ^a	N.D.	N.D.	N.D.	N.D.	3.24 ^a
	95% MeOH	0.90	1.80	1.79	1.57	1.44	2.96	1.58 ^a	2.10 ^a	3.07 ^a	N.D.	3.29 ^a	N.D.	N.D.	2.99 ^a

N.D. : Not detected peak, ^a: Tailing peak, ^b: Low intensity peak

Table 3

The results of reproducibility using final conditions.

Vitamin	Retention time		Area	
	Ave. (min)	%RSD	Ave.	%RSD
A acetate	0.60	0.00	22258	6.66
α -Tocopherol Acetate	0.94	0.47	238678	15.63
K1	1.08	0.51	35306	12.80
K2	1.10	0.50	24617	10.04
A palmitate	1.43	0.00	39479	1.24
α -Tocopherol	1.82	0.00	4035	5.72
β Carotene	2.09	0.26	845	8.40
D2	2.22	0.20	29777	10.51
Nicotinamide	2.35	0.00	110102	3.45
Pyridoxine	2.62	0.00	10904	3.15
Nicotinic acid	2.95	0.15	28420	2.74
D-Pantothenic acid	3.02	0.18	6839	6.21
Riboflavin	3.09	0.14	17878	3.74
Biotin	3.20	0.17	16472	4.37
VC	3.28	0.00	25831	4.64
B12	3.68	0.00	15594	6.72
Thiamine	4.22	0.13	4474072	2.81