1 This document is the Accepted Manuscript version of a Published Work that appeared in final
2 form in Journal of Agricultural and Food Chemistry, copyright © American Chemical Society,
3 after peer review and technical editing by the publisher. To access the final edited and
4 published work see https://pubs.acs.org/doi/10.1021/acs.jafc.9b01617.
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

35

36 **Microwave Assisted Cloud Point Extraction for the Determination of**
37 **Vitamin K Homologues in Vegetables by Liquid Chromatography with**
38 **Tandem Mass Spectrometry**

39

40 **Natalia Campillo[1] • Javier Marín[1] • Pilar Viñas[1] •**
41 **Isabel Garrido[2] • José Fenoll[2] • Manuel Hernández-Córdoba[1]*•**

42

43

44 [1]Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of
45 International Excellence “Campus Mare Nostrum” University of Murcia, E-30100
46 Murcia, Spain

47

48 [2]Equipo de Calidad Alimentaria. Instituto Murciano de Investigación y Desarrollo
49 Agrario y Alimentario (IMIDA). C/ Mayor s/n. La Alberca, 30150 Murcia. Spain

50

51

52

53

54 *Corresponding author:
55 Prof. Manuel Hernández-Córdoba
56 Department of Analytical Chemistry
57 Faculty of Chemistry
58 University of Murcia
59 E-30100 Murcia
60 SPAIN
61 Tel.: +34 868887406
62 FAX: +34 868887682
63 e-mail: hcordoba@um.es

64

ABSTRACT: Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with a triple quadrupole (QqQ) is proposed for determining the vitamin K homologues, phylloquinone (PK), menaquinone-4 (MK) and menadione (MD), in vegetables. The analytes were isolated from the samples (1-1.5 g) by ultrasound assisted extraction using acetonitrile (2 mL), and the liquids were submitted to microwave assisted cloud point extraction with Triton X-45. The enrichment factors were between 20 and 50, depending on the vitamin homologue in question. The analytes were determined by LC-ESI-QqQ-MS/MS in the multiple reaction monitoring (MRM) mode, providing unequivocal identification and quantification, with limits of detection of 0.8, 1.0 and 16 ng/g for MK, PK and MD, respectively. Recovery assays for samples spiked at two concentration levels, between 40 and 600 ng/g depending on the compound, provided recoveries in the 90-114% range. Only PK was detected in the samples analyzed, at concentrations in the 90-2350 ng/g range.

KEYWORDS: cloud point extraction, K vitamins, liquid chromatography-tandem mass spectrometry, menadione, menaquinone, phylloquinone, vegetables

90 **INTRODUCTION**

91 Vitamin K comprises two families of compounds: vitamin K_1 (phylloquinone, PK)
92 and K_2 (menaquinones, MK-n). Both contain a 2-methyl-1,4-naphthoquinone ring
93 in their structure and differ as regard the substituent at the 3-position, which is a
94 phytyl chain for PK and a side chain with repeated isoprenoid units for MK.
95 Whereas PK appears in nature as a single compound, MK comprises a series of
96 vitamers containing from 3 to 14 isoprene units (MK-3 to MK-14).[1,2] PK and MK
97 are the natural forms of vitamin K, whereas menadione (MD, vitamin K_3) is a
98 synthetic derivative used as a pharmaceutical, although the cleavage of dietary
99 PK to produce MD by intestinal bacteria has been described.[3]

100 The role of vitamin K in human health has been reported, being mainly related
101 with blood coagulation and the reduction of osteoporosis and cardiovascular
102 diseases.[1,3] Recent studies show possible perspectives of vitamin K in type 2
103 diabetes treatment, because of its role in glucose metabolism.[4]

104 The K vitamin homologue most commonly found in foods is PK, which is
105 produced by all plants and green algae, where it serves as an electron receptor
106 during photosynthesis. The green color intensity of vegetables is generally
107 related with the PK content, although other variables such as plant growth
108 conditions have also been described.[5,6] Although it has a ubiquitous distribution
109 in the diet, leafy-green vegetables and vegetable oils are the major dietary
110 sources of PK.

111 The synthesis of MK is carried out by bacteria in the intestinal flora, acting as
112 electron carrier in the transport chain involved in respiration.[1] This K homologue
113 occurs in meat, dairy products and fermented foods such as cheese.[7] Interest in
114 MD, which acts as a precursor of vitamin K and as inducer of mitochondrial

115 permeability transitions, has increased in recent years for its activity against
116 cancer cells among other actions.[3]
117 Liquid chromatography (LC) coupled to ultraviolet,[5,6,8-13] and fluorescence[14-21]
118 detection, the latter involving a derivatization step, has been used for vitamin K
119 analysis in a wide variety of samples. The literature also shows the coupling of
120 LC separations to MS and tandem MS (MS/MS) with atmospheric pressure
121 chemical ionization (APCI)[9,10,13,22-27] and electrospray ionization (ESI)[3,28-31], with
122 both ionization sources in positive mode.
123 The food matrices most frequently analyzed for the determination of vitamin
124 K are fruit and vegetable products,[5,6,9,13,15,18,19,23,26,28,30,31] vegetable oils,[6,8,12] milk
125 products and infant formulas.[10,12,14,21,22,29,32] Sample treatment must avoid those
126 conditions affecting the stability of the vitamins, such as ultraviolet light, alkaline
127 and acid media.[2] Food matrices of a lipidic nature such as milk require a
128 hydrolysis step. Thus, neutral lipids have been removed by saponification[10,12,22]
129 or by enzymatic digestion.[21,29] Solvent extraction into organic phases such as
130 hexane to isolate the vitamins from the sample matrix and subsequent purification
131 of the obtained extract by means of solid-phase extraction (SPE) have been
132 widely discussed.[5,15,18,19,23,25,28,30] Matrix solid phase dispersion (MSPD)[9,14] and
133 accelerated solvent extraction (ASE)[23] have been applied to extract K vitamers
134 from different types of food.
135 Miniaturized techniques for sample treatment allow high enrichment factors to
136 be obtained with minimum environmental impact. Dispersive liquid-liquid
137 microextraction (DLLME)[13] and solid-phase microextraction (SPME)[33] have
138 shown their effectiveness for vitamin K preconcentration. Non-ionic surfactant
139 coacervates have been applied extensively in the preconcentration of

140 hydrophobic compounds in a technique known as cloud point extraction (CPE).[34]
141 The use of harmless surfactants, in contrast of toxic and flammable organic
142 solvents commonly implied in conventional extraction methods, place CPE as a
143 green microextraction procedure of high value. In the case of MD, the possibilities
144 of using CPE were preliminarily explored by spectrophotometric determination[35]
145 and for PK using LC-UV detection,[36] the latter without quantification purposes.
146 Considering the advantageous characteristics of CPE for limiting the use of
147 organic solvents in the preconcentration step, a new procedure is described here
148 and optimized for the determination of PK, MK and MD in vegetables by LC-ESI-
149 QqQ-MS/MS.

150

151 **MATERIALS AND METHODS**

152 **Reagents and materials.** Phylloquinone (PK, vitamin K_1), menaquinone-4
153 (MK-4, vitamin K_2) and menadione (MD, vitamin K_3) were purchased from Sigma-
154 Aldrich (St. Louis, MO, USA). Individual stock solutions (1000 μg/mL) of PK, MK-
155 4 and MD were prepared in ethanol, preventing photodegradation of the vitamins
156 by storing the stocks solutions in amber vials at -20 ºC. The stability of these
157 solutions was checked for at least one month. Working standard solutions were
158 prepared daily in ethanol and stored at 4 ºC. The non-ionic surfactant
159 polyethylene glycol 4-*tert*-octylphenyl ether (Triton X-45), provided by Fluka
160 (Buchs SG, Switzerland), was used as a 15% w/v aqueous solution. Other
161 assayed surfactants were *tert*-octylphenoxypolyethoxyethanol (Triton X-100) and
162 octylphenoxypoly-ethoxyethanol (Triton X-114). Sodium chloride (99.5% purity),
163 ammonium acetate and formic acid were obtained from Fluka. The 5 mol/L
164 ammonium formate solution (Agilent, Waldbronn, Germany) was appropriately

165 diluted and used in the mobile phase. Methanol, ethanol, acetonitrile and
166 isopropanol were obtained from Sigma.

167 **Instrumentation.** An Agilent 1200 (Agilent) quaternary pump (G1311A)
168 equipped with a chromatographic column thermostatic compartment (Agilent
169 1200, G1316B) was operated as indicated in Table 1. An autosampler was used
170 for LC injection, solutions being maintained in 250 µL microinserts with polymeric
171 feet placed into vials of 2 mL capacity. An Agilent G6410A mass spectrometer
172 was coupled to the LC system (Table 1). Mass spectra were monitorized in the
173 50-700 amu *m/z* range. For data acquisition, instrument control, identification of
174 the vitamins as well as their quantitation the Agilent MassHunter software was
175 used.

176 Individual analyte solutions (10 µg/mL) were directly infused into the ion
177 source, applying various fragmentor voltages and collision energies (Table 2), in
178 order to identify the optimal multiple reaction monitoring (MRM) transitions for
179 each vitamin. Identification of the vitamins was done using retention time and
180 MRM transitions involving the formation of product ions with highest *m/z* value.
181 The transition of higher sensitivity was used as quantifier, and all transitions were
182 used as qualifier peaks for confirmatory analysis (Table 2).

183 An IKA A11 homogenizer (IKA Works, INC., Wilmington, NC, USA), an UP
184 200 H ultrasonic processor (Dr. Hielscher, Teltow, Germany) equipped with a
185 titanium sonotrode (7 mm i.d.) and providing in liquid media an effective output of
186 200 W, and an EBA 20 centrifuge (Hettich, Tuttlingen, Germany) were used in
187 the sample treatment. A domestic microwave oven was used for the formation of
188 the cloudy solution.

189 **Samples and analytical procedures.** A total of ten different vegetables
190 (iceberg lettuce, romaine lettuce, lamb´s lettuce, escarole lettuce, kale, spinach,
191 cress, turnip, parsnip and carrot) were obtained from local supermarkets. The
192 samples were washed with distilled water and allowed to dry to remove excess
193 water. To prevent deterioration of the K vitamins, samples were maintained in
194 subdued light at 4 ºC until analysis, which was generally carried out within 48 h
195 of arrival in the laboratory. All samples were manually chopped into pieces of
196 about 0.5 cm and homogenized using an electric homogenizer.

197 Ultrasound assisted extraction (UAE) was applied to isolate vitamins from the
198 solid matrices. For this, a 1-1.5 g aliquot of sample (depending on the vitamin
199 content) was weighed into a 15 mL glass centrifuge containing 0.15 g sodium
200 chloride, and 2 mL of acetonitrile were added. The mixture was sonicated by
201 means of a probe directly immersed for 1 min with 0.75 s pulses of 105 µm
202 amplitude and then centrifuged at 6000 rpm for 5 min. A 1 mL volume of the
203 acetonitrile supernatant was recovered and diluted with 5 mL of 10 mM
204 ammonium acetate (pH 7) in the presence of 0.04% w/v sodium chloride. Next,
205 50 µL of a 15% w/v Triton X-45 aqueous solution was injected into the sample
206 and the mixture was shaken manually for a few seconds. The resulting solution
207 was placed in a water bath and heated in a domestic microwave oven working at
208 maximum power for 20 s, leading to the formation of a cloudy solution. The K
209 vitamins were extracted into the fine droplets of the coacervate dispersed through
210 the sample solution. The tube was then immersed into an ice bath for 5 min in
211 order to favour the separation of the surfactant rich phase, which sedimented at
212 the bottom of the conical tube (drop volume of about 15 µL) and was collected
213 using a microsyringe. The recovered drop was placed in a microvial and diluted

214 by adding 30 µL of methanol, before being injected (20 µL) into the LC system by
215 means of an autosampler.

216 As a positive quality control (QC) sample, a mixture of PK, MK and MD at 50,
217 50 and 100 ng/mL levels, respectively, prepared in ethanol was used. This
218 standard mixture was analysed before each sample batch and stored at -20 ºC
219 between analysis, and its stability was verified for one month. An appropriate
220 blank sample (parsnip) was also used as a QC sample.

221 Calibration standards were prepared by adding different volumes in the 10-
222 300 µL range of a standard solution containing 1 µg/mL of PK and MK and 10
223 µg/mL of MD to 6 mL of a 1:5 acetonitrile:water mixture.

224 **Recovery studies.** For recovery studies, 1-1.5 g aliquots of the homogenized
225 samples were fortified by adding different volumes in the 50-225 µL range of an
226 ethanolic standard solution containing the vitamins (0.8 µg/mL for PK and MK
227 and 4 µg/mL for MD), leading to fortification levels in the 40-120 ng/g range for
228 PK and MK, and in the 200-600 ng/g for MD. The fortified samples were
229 homogenized for 5 min and left to stand for 1 h at room temperature to distribute
230 the analytes evenly and to allow them to interact with the matrix, before carrying
231 out the analyses as described above. The fortification procedure was applied to
232 two different samples (iceberg lettuce and turnip). Three aliquots of each sample
233 at each concentration were analysed separately. Recovery percentages were
234 also obtained when samples were spiked after application of the UAE and CPE
235 steps.

236 **Precision study.** For precision study, 1.5 g aliquots of homogenized parsnip
237 were fortified by adding 100 µL of an ethanolic standard solution containing the
238 vitamins at 1.5 µg/mL for PK and MK and 3 µg/mL for MD, leading to fortification

239 levels of 100 ng/g for PK and MK and 200 ng/g for MD. The fortified aliquots were
240 homogenized for 5 min and left to stand form 1 hour at room temperature, before
241 carrying out their analysis, in order to stablish the intra- and inter-day precision
242 method.
243
244
245 **RESULTS AND DISCUSSION**

246 **Chromatographic separation.** Liquid chromatography in the reversed phase
247 mode was used, optimizing the separation conditions by injecting 20 μL of an
248 ethanolic standard solution containing the analytes at 2 μg/mL concentration
249 level. Different mixtures of solvent A (0.1% v/v formic acid and 10 mM ammonium
250 formate aqueous solution) and solvent B (0.1% v/v formic acid and 10 mM
251 ammonium formate methanolic solution) were tested. The finally selected
252 conditions for the chromatographic separation, the retention time of each vitamin,
253 as well as the MS/MS detection conditions are shown in Table 1. The retention
254 times showed standard deviations, depending on the compound, in the 0.02-0.06
255 min range for 10 successive analysis carried out on the same day and between
256 0.03 and 0.16 min range, for 10 successive analyses carried out on different days.

257 **Optimization of the ultrasound extraction step.** Food sample preparation
258 for liposoluble vitamins analysis generally involves saponification, solid-liquid and
259 liquid-liquid extractions, sometimes including enzymatic hydrolysis.[37] The
260 instability of K vitamins under alkaline conditions prevents from being applied the
261 very effective caustic saponification process used in the determination of other
262 liposoluble vitamins to effectively remove interferences of lipid origin contained in
263 the sample matrix. Consequently, a direct solid-liquid extraction in acetonitrile

264 was adopted since this has proven effective for extracting K vitamins from
265 vegetable matrices.[13] Moreover, taking into account the influence of light and heat
266 on K vitamin stability, sample treatment was carried out at room temperature and
267 in dim light.

268 This step was optimized using romaine lettuce (fortified for MD and MK at a
269 0.5 μg/g concentration level), testing different sample masses in the 1-3 g range
270 and different acetonitrile volumes of between 1 and 3 mL. The mixtures were
271 homogenized using an orbital shaker for 5 min at maximum power and then
272 centrifuged for 5 min at 3000 rpm. The highest signals were obtained for a sample
273 mass of 1.5 g extracted with 2 mL of acetonitrile, a ratio that was selected for
274 further experiments.

275 The effect of the application of ultrasounds for 5 min using a water bath
276 operating at a frequency of 40 kHz, and by means of a probe directly immersed
277 for 1 min into the sample:acetonitrile mixture was compared. Peak areas obtained
278 for the three vitamins using the ultrasounds probe were about four times higher
279 than those obtained when using the water bath. Thus, UAE by means of the probe
280 was selected, proving its effectiveness for extracting vitamin K congeners in
281 acetonitrile from vegetable samples. The application of ultrasounds for longer
282 times did not increase extraction efficiency.

283 The addition of sodium chloride to the sample:acetonitrile mixture increased
284 the transfer of the vitamins towards the organic phase, and also provided a good
285 separation of the phases. When different NaCl masses in the 0.1-0.5 g range
286 were assayed, best results were obtained for 0.15 g of salt, while sensitivity
287 decreased for higher masses. Consequently, 0.15 g NaCl was selected.

288 **Preconcentration procedure optimization.** Preliminary experiments for
289 preconcentrating the vitamins were carried out using DLLME with ionic liquids
290 (IL-DLLME) as extractant solvent. For such a purpose, the extraction efficiency
291 of 1-hexyl-3-methylimidazolium $[C_6MIm][NTf_2]$, 1-methyl-3-octylimidazolium
292 $[C_8MIm][NTf_2]$ and 1-dodecyl-3-methylimidazolium $[C_{12}MIm][NTf_2]$
293 bis(trifluoromethylsulfonyl)imide was tested using 100 µL of the IL, 0.5 mL of
294 acetonitrile as dispersant solvent and 10 mL of aqueous phase containing the
295 analytes at 100 ng/mL. The IL enriched phase (25-30 µL) was recovered by
296 centrifugation and diluted by adding 30 µL of methanol in order to decrease its
297 viscosity. The *in situ* formation of $[C_6MIm][NTf_2]$ IL was also tried by adding 50 µL
298 of the IL ionic parts but no significant differences were observed compared with
299 the addition of the previously formed IL. The application of the IL-DLLME
300 procedure was discarded because the extraction capacity for the different ILs was
301 in all cases about one third of that provided by CPE (Figure 1), the latter being
302 adopted because it avoided the use of organic toxic solvents.
303 Triton X-45, Triton X-114 and Triton X-100 were assayed as extractants for
304 CPE, using in the preliminary experiments, volumes of 100 µL of 15% w/v
305 solutions of the surfactants, and heating the mixture up to the corresponding
306 cloud point temperatures for 10 min in a bath water. No discernible surfactant
307 enriched phase was obtained when Triton X-100 was used, and so this extractant
308 was discarded. The extraction efficiencies of Triton X-45 and Triton X-114 are
309 shown in Fig. 1, where it can be observed that the highest sensitivity was
310 achieved using Triton X-45, which was selected.
311 The different experimental variables affecting the extraction efficiency of CPE
312 were optimized by means of a Taguchi orthogonal array design[38] applied for four

factors (each one considered at three different levels), namely, surfactant volume, aqueous phase volume, sodium chloride concentration and heating time (Table S1). For this purpose, 1 mL of the acetonitrile extract obtained in the UAE step applied to romaine lettuce was fortified with MK and MD at 300 ng/mL and diluted with different water volumes in the 5-9 mL range. The volume of the surfactant (acceptor) phase was between 50 and 150 µL. The effect of the aqueous phase ionic strength was studied in the 0.2 - 0.4 g/L sodium chloride concentration range. When the surfactant-donor phase mixture was heated above the corresponding cloud point temperature (in the 25-38 ºC depending on the concentration),[39] the formation of a coacervate resulted in a cloudy solution. The heating time was studied in the 10-20 min range using a water bath. In all cases, centrifugation of the mixtures at 3500 rpm for 3 min, and a subsequent cooling step at -20 ºC for 5 min, were applied in order to facilitate phase separation. A 20 µL volume of the surfactant enriched phase previously diluted with methanol was injected into the liquid chromatograph. The effects of the four factors studied on the mean response for the extraction efficiency of K vitamins showed that the best results were obtained using both the lowest surfactant and lowest aqueous phase volumes (Fig. S1). A 50 µL volume of Triton X-45 minimized the dilution effect. The high extraction efficiency of CPE even at low surfactant concentrations is here corroborated.[34] When lower volumes than 50 µL were assayed, collecting the enriched phase was very difficult owing to the minimal amount recovered. Peak area of the vitamins was maximum for a salt level of 0.4 g/L. From a practical point of view, it is more convenient to replace the heating step by a microwave oven in order to shorten the analysis time.[40] In this way, even higher sensitivity than that attained using the water bath was

338 obtained by heating the mixture in a domestic microwave oven for 20 s, which
339 corresponded to about 50 ºC.

340 The influence of the pH of the donor phase was studied by using 5 mL of 0.01
341 M buffer solutions of pH values ranging from 3 to 9 as aqueous phases. The
342 sensitivity of the three vitamins increased up to pH 7, and then slightly decreased
343 for higher pH values. Consequently, a pH of 7 was selected. Note that the
344 surfactant-rich phase volume recovered (15 μL) was diluted by adding 30 μL
345 methanol in order to reduce the viscosity of the injected solution. Thus, CPE was
346 seen to effectively preconcentrate the K vitamins using a rapid, green and easy
347 to apply procedure.

348 **Method performance.** An ANOVA test was applied to compare the slopes of
349 standard calibration graphs and those obtained when the standard additions
350 method was applied to three different samples in order to check the relevance of
351 any matrix effect. Since in the three samples studied "p" values higher than 0.05
352 were obtained for the three vitamins, the lack of a matrix effect was confirmed.
353 Consequently, the external standard procedure was used for quantification
354 purposes. For this, standards prepared in acetonitrile:water (1:5) were submitted
355 to the optimized CPE preconcentration step before being injected into the LC
356 system.

357 CPE combined with the LC-ESI-QqQ-MS/MS method was validated for
358 linearity, limits of detection (LODs), limits of quantification (LOQs) and
359 repeatability. Calibration curves were obtained by least-squares linear regression
360 analysis of the peak area *vs.* analyte concentration using six concentration levels.
361 Linearities ranged between 15 and 500 ng/mL for MD and between 1 and 500
362 ng/mL for MK and PK. Calibration slope values expressed as mean value ±

363 standard deviation (n=6) of 59±4, 894±49 and 825±71 mL/ng were obtained for
364 MD, MK and PK, respectively. Regression coefficients higher than 0.9970 were
365 obtained in all cases. LODs and LOQs were calculated on the basis of three and
366 ten times the standard deviation of the intercept of the calibration graphs,
367 respectively. LODs and LOQs of 4 and 13, 0.2 and 0.7 and 0.25 and 0.8 ng/mL
368 were obtained for MD, MK and PK, respectively. Considering the sample
369 procedure applied in the vegetable analysis, LODs and LOQs varied in the 0.8-
370 16 and 2.8-52 ng/g ranges, depending on the compound. The LOD value
371 obtained for PK (0.8 ng/g) allows its detection in vegetables for which a
372 consumption of 100 g contributes 0.1% of the adequate ingestion set for an adult
373 person by European Food Safety Authority (EFSA) panel on dietetic products,
374 nutrition and allergies of 1 μg/kg body weight per day.[41]

375 A precision study was carried out based on the repeatability and
376 reproducibility, calculated as the relative standard deviation (RSD) for a series of
377 ten analyses carried out on the same day and in different days, of a parsnip
378 sample fortified at 100 ng/g with PK and MK and 200 ng/g with MD. RSD values
379 of 8.8 and 9.2% for PK, 5.1 and 6.3% for MK and 10.5 and 10.9% for MD were
380 found for intra- and inter-day precision, respectively. These data indicate the
381 satisfactory degree of precision of the developed method.

382 The ratio between slopes of the calibration curves obtained for the CPE
383 method and those obtained without using the preconcentration step was used to
384 calculate the enrichment factors (EFs), values of 50, 45 and 20 (corresponding
385 to PK, MK and MD, respectively) being obtained. These EF values indicated that
386 PK and MK were most efficiently preconcentrated by the CPE method developed,

387 whereas MD showed lower affinity for extraction into the coacervate, probably
388 due to its lacking a side chain at the C-3 position, unlike PK and MK.

389 A comparison of the developed method with previously published methods for
390 determining K vitamins in food samples by LC is shown in Table 3. Note that
391 CPE is used for the first time to preconcentrate the K vitamins, providing, in the
392 case of PK and MK, comparable sensitivities to those previously obtained using
393 conventional sample treatments such as SPE[18,23,25,30] or the miniaturized
394 procedures DLLME[13] and MSPD,[14] in a simple and rapid treatment and avoiding
395 the use of toxic organic solvents. The analytical characteristics obtained show
396 that UAE-CPE combined with LC-ESI-QqQ-MS/MS can be used for the routine
397 analysis of vegetables, minimizing sample handling.

398 **Analysis of samples and recovery studies.** CPE combined with the optimized
399 LC-ESI-QqQ-MS/MS procedure was applied to the analysis of ten different
400 vegetables in triplicate. PK contents in the 90-2350 ng/g range were obtained for
401 iceberg and lamb´s lettuce, respectively (Table 4). MK and MD were not detected
402 in the studied samples, and none of the K vitamins under analysis was contained
403 in the samples of parsnip, turnip and carrot, at least above their corresponding
404 LODs. A second treatment was made to verify that extraction was completed. As
405 expected, green-leaf vegetables were confirmed to be good sources of vitamin
406 K, whereas it was absent in the analysed root vegetables.

407 Fig. 2 shows the total ion chromatograms (TIC) obtained for a standard
408 mixture solution (40 ng/mL for PK and MK and 80 ng/mL for MD) and for a spinach
409 sample using the optimized method.

410 The reliability of the procedure was checked by recovery studies for two
411 different samples spiked at three concentration levels. The recoveries of the

412 vitamins from spiked samples (turnip and iceberg lettuce) varied between 90.3
413 and 114%, with an average recovery ± SD (n = 18) of 101 ± 7 (Table S2), when
414 samples were fortified before being submitted to UAE. Recovery values in the
415 93.2-109.8% range were obtained when comparing peak areas obtained when
416 the target analytes were spiked before and after the UAE-CPE procedure. No
417 significant differences were observed in the RSD values obtained for the
418 recoveries of the three vitamins in each fortified sample, the values ranging
419 between 3.9 and 8.4%. The analytes were identified using their retention times,
420 the transitions given in the mass spectra and by comparing the percentage for
421 each transition obtained from standard solutions and samples. No interfering
422 peaks were observed at the analyte elution times.

423

424 **Acknowledgements**

425 The authors acknowledge the financial support of the Comunidad Autónoma de
426 la Región de Murcia (Fundación Séneca, 19888/GERM/15), the Spanish
427 MINECO (Project PGC2018-098363-B-100) and the European Commission
428 (FEDER/ERDF). They are also grateful to Juana Cava for technical assistance.

429

430 **Declaration of interest**

431 The authors declare no competing financial interest.

432

433 **ABBREVIATIONS USED**

434 ASE, accelerated solvent extraction; APCI, atmospheric pressure chemical
435 ionization; CPE, cloud point extraction; DLLME, dispersive liquid-liquid
436 microextraction; ESI, electrospray ionization; LC, liquid chromatography; LOD,

437 limit of detection; LOQ, limit of quantification; MD, menadione; MK,
438 menaquinone-4; MRM, multiple reaction monitoring; MS, mass spectrometry;
439 MS/MS, tandem mass spectrometry; MSPD, matrix solid phase dispersion; PK,
440 phylloquinone; QqQ, triple quadrupole; RSD, relative standard deviation; SD;
441 standard deviation; SPE, solid-phase extraction; SPME, solid-phase
442 microextraction; UAE, ultrasound assisted extraction

443

444 **REFERENCES**

445 (1) Berenjian, A.; Mahanama, R.; Kavanagh, J.; Dehghani, F. Vitamin K
446 series: current status and future prospects. *Crit. Rev. Biotechnol.* **2015**, *35*, 199–
447 208.
448 (2) Fanali, C.; D'Orazio, G.; Fanali, S.; Gentili, A. Advanced analytical
449 techniques for fat-soluble vitamin analysis. *TrAC, Trends Anal. Chem.* **2017**, *87*,
450 82–97.
451 (3) Liu, R.; Wang, M.; Ding, L. A novel liquid chromatography-tandem mass
452 spectrometry method for determination of menadione in human plasma after
453 derivatization with 3-mercaptopropionic acid. *Talanta* **2014**, *128*, 51–57.
454 (4) Bourron, O.; Phan, F. Vitamin K: a nutrient which plays a little-known role
455 in glucose metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **2019**, *22*, 174–181.
456 (5) Kim, M.; Chiu, Y.; Ku, K. Glucosinolates, carotenoids, and vitamins E and
457 K variation from selected kale and collard cultivars. *J. Food Quality* **2017**,
458 *5123572*, 1–8.
459 (6) Otles, S.; Cagindi, O. Determination of vitamin K1 content in olive oil, chard
460 and human plasma by RP-HPLC method with UV–Vis detection. *Food Chem.*
461 **2007**, *100*, 1220–1222.

462 (7) Booth, S. L. Vitamin K: food composition and dietary intakes. *Food Nutr.*
463 *Res.* **2012**, *56*, 5505–5509.
464 (8) Carballo, S.; Prats, S.; Maestre, S.; Todoli, J. Determination of fat-soluble
465 vitamins in vegetable oils through microwave-assisted high-performance liquid
466 chromatography. *J. Sep. Sci.* **2015**, *38*, 1073–1081.
467 (9) Gentili, A.; Caretti, F. Evaluation of a method based on liquid
468 chromatography–diode array detector–tandem mass spectrometry for a rapid
469 and comprehensive characterization of the fat-soluble vitamin and carotenoid
470 profile of selected plant foods. *J. Chromatogr. A* **2011**, *1218*, 684–697.
471 (10) Gentili, A.; Caretti, F.; Bellante, S.; Ventura, S.; Canepari, S.; Curini,
472 R. Comprehensive profiling of carotenoids and fat-soluble vitamins in milk from
473 different animal species by LC-DAD-MS/MS hyphenation. *J. Agric. Food Chem.*
474 **2013**, *61*, 1628–1639.
475 (11) Gomis, D.; Fernandez, M.; Gutiérrez Alvarez, M. Simultaneous
476 determination of fat-soluble vitamins and provitamins in milk by microcolumn
477 liquid chromatography. *J. Chromatogr. A* **2000**, *891*, 109–114.
478 (12) Kienen, V.; Costa, W.; Visentainer, J.; Souza, N; Oliveira, C. C.
479 Development of a green chromatographic method for determination of fat-soluble
480 vitamins in food and pharmaceutical supplement. *Talanta* **2008**, *75*, 141–146.
481 (13) Viñas, P.; Bravo-Bravo, M.; López-García, I.; Hernández-Córdoba,
482 M. Dispersive liquid–liquid microextraction for the determination of vitamins D and
483 K in foods by liquid chromatography with diode-array and atmospheric pressure
484 chemical ionization-mass spectrometry detection. *Talanta* **2013**, *115*, 806–813.
485 (14) Chase, G.; Eitenmiller, R.; Long, A. The liquid chromatographic
486 analysis of vitamin K1 in soy based infant formula using matrix solid phase

487 dispersion. *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 423–432.

488 (15) Damon, M.; Zhang, N.; Haytowitz, D.; Booth, S. L. Phylloquinone
489 (vitamin K1) content of vegetables. *J. Food Compos. Anal.* **2005**, *18*, 751–758.

490 (16) Iwase, H. Determination of vitamin K_1 in emulsified nutritional
491 supplements by solid-phase extraction and high-performance liquid
492 chromatography with postcolumn reduction on a platinum catalyst and
493 fluorescence detection. *J. Chromatogr. A* **2000**, *881*, 261–266.

494 (17) Kamao, M.; Suhara, Y.; Tsugawa, N.; Okano, T. Determination of
495 plasma Vitamin K by high-performance liquid chromatography with fluorescence
496 detection using Vitamin K analogs as internal standards. *J. Chromatogr. B* **2005**,
497 *816*, 41–48.

498 (18) Pérez-Ruiz, T.; Martínez-Lozano, C.; García, M.; Martín, J. High-
499 performance liquid chromatography–photochemical reduction in aerobic
500 conditions for determination of K vitamins using fluorescence detection. *J.*
501 *Chromatogr. A* **2007**, *114*, 67–72.

502 (19) Presse, N.; Potvin, S.; Bertrand, B.; Calvo, M.; Ferland, G.
503 Phylloquinone content of herbs, spices and seasonings. *J. Food Compos. Anal.*
504 **2015**, *41*, 15–20.

505 (20) Ware, G.; Chase, G.; Eitenmiller, R.; Long, A. Determination of
506 vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction
507 and fluorometric detection. *J. AOAC Int.* **2000**, *83*, 957–962.

508 (21) Woollard, D. C.; Indyk, H. E.; Fong, B. Y.; Cook, K. K. Determination
509 of vitamin K_1 isomers in foods by liquid chromatography with C-30 bonded-phase
510 column. *J. AOAC Int.* **2002***,* 85, 682–691.

511 (22) Gentili, A.; Miccheli, A.; Tomai, P.; Baldassarre, M.; Curini, R.;

512 Pérez-Fernández, V. Liquid chromatography–tandem mass spectrometry
513 method for the determination of vitamin K homologues in human milk after
514 overnight cold saponification. *J. Food Compos. Anal.* **2016**, *47*, 21–30.
515 (23) Jäpelt, R.; Jakobsen, J. Analysis of vitamin K_1 in fruits and
516 vegetables using accelerated solvent extraction and liquid chromatography
517 tandem mass spectrometry with atmospheric pressure chemical ionization. *Food*
518 *Chem.* **2016**, *192*, 402–408.
519 (24) Kamao, M.; Hirota, Y.; Suhara, Y.; Tsugawa, N.; Nakagawa, K.;
520 Okano, T.; Hasegawa, H. Determination of menadione by liquid
521 chromatography–tandem mass spectrometry using pseudo multiple reaction
522 monitoring. *Anal. Sci.* **2017**, *33*, 863–867.
523 (25) Karl, J.; Fu, X.; Dolnikowski, G.; Saltzman, E.; Booth, S. L.
524 Quantification of phylloquinone and menaquinones in feces, serum, and food by
525 high-performance liquid chromatography–mass spectrometry. *J. Chromatogr. B*
526 **2014**, *963*, 128–133.
527 (26) Kurilich, A.; Britz, S.; Clevidence, B.; Novotny, J. Isotopic labeling
528 and LC-APCI-MS quantification for investigating absorption of carotenoids and
529 phylloquinone from kale (Brassica oleracea). *J. Agric. Food Chem.* **2003**, *51*,
530 4877–4883.
531 (27) Fu, X. Y.; Shen, X. H.; Finnan, E. G.; Haytowitz, D. B.; Booth, S. L.
532 Measurement of multiple vitamin K forms in processed and fresh-cut pork
533 products in the US food supply. *J. Agric. Food Chem.* **2016**, *64*, 4531–4535.
534 (28) Claussen, F.; Taylor, M.; Breeze, M.; Liu, K. Measurement of
535 vitamin K1 in commercial canola cultivars from growing locations in North and
536 South America using high-performance liquid chromatography–tandem mass

537 spectrometry. *J. Agric. Food Chem.* **2015**, *63*, 1076–1081.

538 (29) Huang, B.; Zheng, F.; Fu, S.; Yao, J.; Tao, B.; Ren, Y. UPLC-ESI-
539 MS/MS for determining trans- and cis-vitamin K_1 in infant formulas: method and
540 applications. *Eur. Food Res. Technol.* **2012**, *235*, 873–879.

541 (30) Huang, B.; Wang, Z.; Yao, J.; Ke, X.; Xu, J.; Pan, X.; Xu, X.; Lu, M.;
542 Ren, Y. Quantitative analysis of vitamin K_1 in fruits and vegetables by isotope
543 dilution LC-MS/MS. *Anal. Methods* **2016**, *8*, 5707–5711.

544 (31) Tayade, A.; Dhar, P.; Kumar, J.; Sharma, M.; Chaurasia, O.;
545 Srivastava, R. Sequential determination of fat- and water-soluble vitamins in
546 Rhodiola imbricata root from trans-Himalaya with rapid resolution liquid. *Anal.*
547 *Chim. Acta* **2013**, *789*, 65–73.

548 (32) Oberson, J.; Campos-Giménez, E.; Rivière, J.; Martin, F.
549 Application of supercritical fluid chromatography coupled to mass spectrometry
550 to the determination of fat-soluble vitamins in selected food products. *J.*
551 *Chromatogr. B* **2018***, 1086*, 118–129.

552 (33) Reto, M.; Figueira, M. E.; Filipe, H. M.; Almeida, C. M. M. Analysis
553 of vitamin K in green tea leafs and infusions by SPME–GC-FID. *Food Chem.*
554 **2007**, *100*, 405–411.

555 (34) Ballesteros-Gómez, A.; Sicilia, M.; Rubio, S. Supramolecular
556 solvents in the extraction of organic compounds. A review. *Anal. Chim. Acta*
557 **2010**, *677*, 108–130.

558 (35) Abdollahi, H.; Bagheri, L. Simultaneous spectrophotometric
559 determination of vitamin K3 and 1,4-naphthoquinone after cloud point extraction
560 by using genetic algorithm. *Anal. Chim. Acta* **2004**, *514*, 211–218.

561 (36) Cordero, B. M.; Pavón, J. L. P.; Pinto, C. G.; Laespada, M. E. F.

562 Cloud point methodology: A new approach for preconcentration and separation
563 in hydrodynamic systems of analysis. *Talanta* **1993**, *40*, 1703–1710.

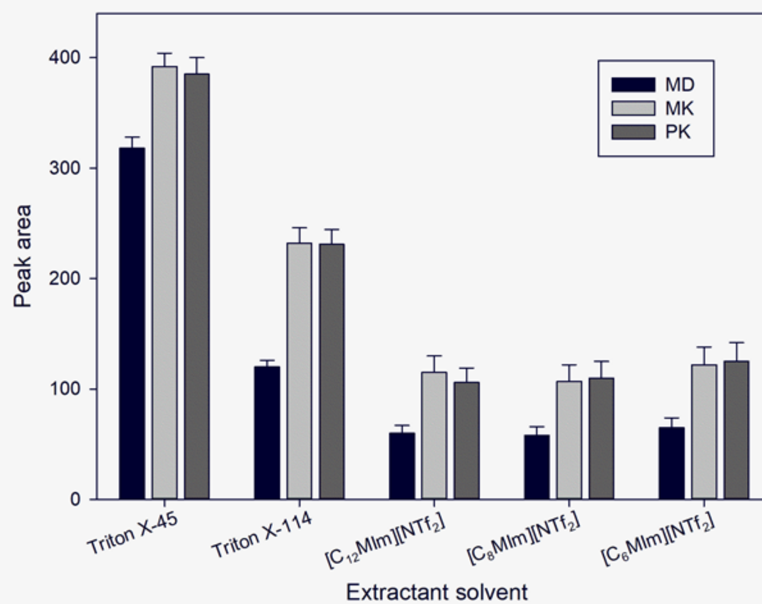
564 (37) Cortés-Herrera, C.; Artavia, G.; Leiva, A.; Granados-Chinchilla, F.
565 Liquid chromatography analysis of common nutritional components, in feed and
566 food. *Foods* **2019**, *8*, 1-63.

567 (38) Peace, G. S. Taguchi methods: a hands-on approach, Addison-
568 Wesley, Reading, MA, 1993.

569 (39) Tan, G. Y. T.; Zimmermann, W.; Lee, K.; Lan J. C.; Yim, H. S.; Ng,
570 H. S. Recovery of mangostins from Garcinia Mangostana peels with an aqeuous
571 micellar biphasic system. *Food Bioprod. Process* **2017**, *102*, 233-240.

572 (40) Cacho, J. I.; Campillo, N.; Viñas, P.; Hernández-Córdoba, M. Cloud
573 point extraction and gas chromatography with direct microvial insert thermal
574 desorption for the determination of haloanisoles in alcoholic beverages. *Talanta*
575 **2016**, *160*, 282–288.

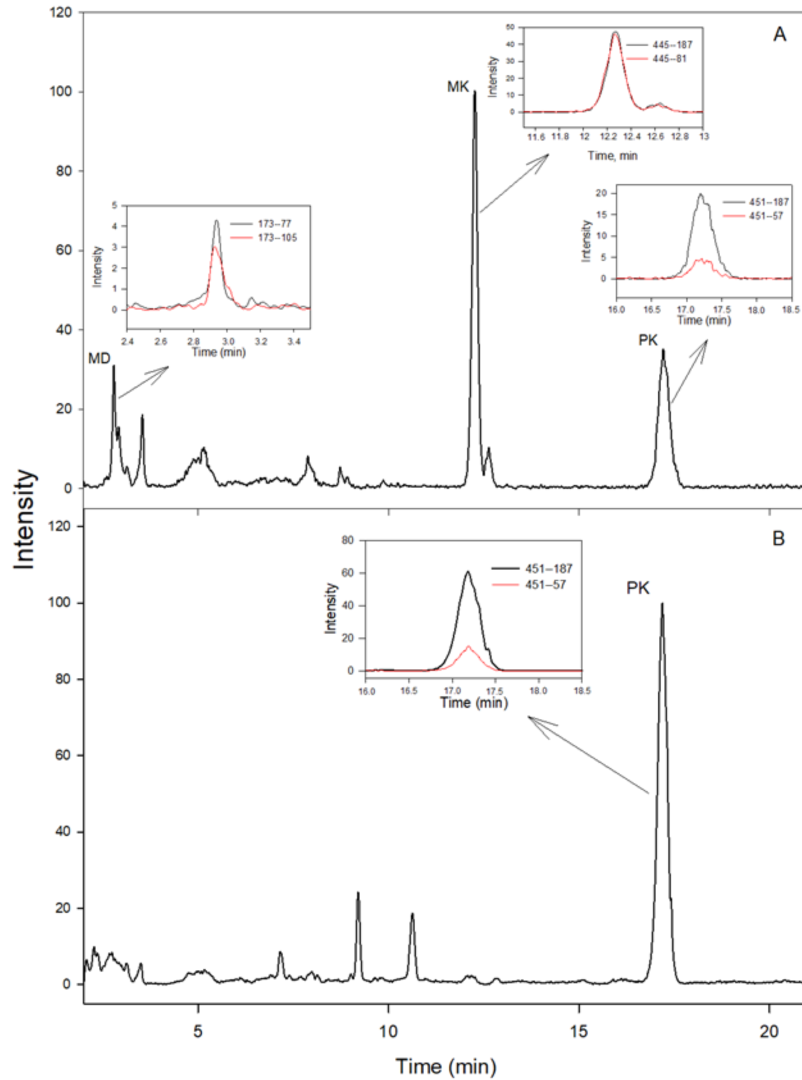
576 (41) European Food Safety Authority EFSA Panel on dietetic products,
577 nutrition and allergies. Dietary reference values for vitamin K. *EFSA J.*, **2017**, *15*,
578 4780–4858.



579

580

581 **Figure 1.** Effect of the extractant phase nature on the sensitivity of the
582 microextraction procedure. Vertical lines indicate standard deviation for n=3.



583

584

585 **Figure 2.** Total ion chromatograms of K vitamins obtained for a standard mixture

586 (40 ng/mL for PK and MK and 80 ng/mL for MD) (A) and an un-spiked spinach

587 sample (B) using CPE combined with LC-ESI-QqQ-MS/MS under the optimized

588 conditions.

589

590

591

592

593

Table 1. Experimental Conditions Of The LC-ESI-QqQ-MS/MS System

LC separation	
analytical column	Tracer Extrasil ODS2 (150 x 4 mm, 5 µm particle size)
column temperature	35 ºC
injection volume	20 µL
mobile phase composition	solvent A: 0.1% v/v formic acid and 10 mM ammonium formate aqueous solution solvent B: 0.1% v/v formic acid and 10 mM ammonium formate methanolic solution
mobile phase gradient	0-3 min, 10:90 A:B 4 min, 100% B (maintained for 13 min) 18 min, 10:90 A:B (maintained for 3 min)
mobile phase flow-rate	0.6 mL/min
MS/MS detection	
ionization system conditions	ESI, positive mode; Capillary voltage, 3000 V; nebulizer pressure, 40 psi; drying gas flow, 9 L/min; drying gas temperature, 350 ºC
nebulizer and collision gas	nitrogen
analyzer	triple quadrupole

594

Table 2. Molecular Formula and Analytical Conditions of the Studied K Vitamins

vitamin	molecular formula	molecular weight	t_R (min)	ionization mode	target ion transitions (*m/z*)	fragmentor voltage (V)	collision energy (V)	abundance ratio (%)
menadione (MD)	$C_{11}H_8O_2$	172.18	2.85	$[M+H]^+$	173[a] → 77	110	40	
					173 → 105	110	20	83
					173 → 42	110	50	42
menaquinone (MK-4)	$C_{31}H_{40}O_2$	444.65	12.24	$[M+H]^+$	445[a] → 187	130	15	
					445 → 81	130	40	99
					445 → 363	130	5	12
phylloquinone (PK)	$C_{31}H_{46}O_2$	450.70	17.20	$[M+H]^+$	451[a] → 187	140	20	
					451 → 57	140	40	22
					451 → 185	140	20	17

[a] Quantifier transition.

Table 3. Comparison With Other Methods Proposed For K Vitamin Analysis In Foods

compound	sample			detection technique	LOD (ng/g)	ref
	nature	treatment	mass (g)			
PK, MK	maize flour, kiwi	MSPD	2.0	LC-DAD-APCI-MS/MS	PK: 0.4-18 MK: 1-5	10
PK	fruits, vegetables	ASE-SPE	0.1-1.3	LC-APCI-MS/MS	1.5	23
PK	fruits, vegetables	UAE-SPE	5	ID-LC-ESI-MS/MS	1.0	30
PK	kale, collard	SLE-SPE	0.3	LC-DAD	Not provided	5
PK	herbs, spices, seasonings	UAE-SPE	0.05-0.2	LC-FLD	Not provided	19
PK, MK	*Rhodiola imbricata* root	UAE	1	RRLC-ESI-MS/MS	4.1; 2.1	31
PK, MK, MD	vegetables	UAE-SPE	1	LC-FLD	1.4; 0.6; 0.8	18
PK, MK, MD	vegetables	SLE-DLLME	0.2-2	LC-DAD-APCI-MS	DAD: 0.45; 0.45; 0.3 MS: 0.45; 0.3; 0.75	13
PK	soy-based infant formula	MSPD	0.5	LC-FLD	27	14
PK, MK	baby foods	SLE-SPE	0.2	LC-APCI-MS	13.5; 13.3	25
PK	milk, soy bean oil	alkaline digestion-LLE	30	LC-DAD	138	12
PK, MK, MD	vegetables	UAE-CPE	1.5	LC-ESI-MS/MS	MS/MS: 1.0; 0.8; 16	this method

ASE, accelerated solvent extraction; FLD, fluorescence detection; ID, isotopic dilution; MSPD, matrix solid phase dispersion; RRLC, rapid resolution liquid chromatography

Table 4. PK Contents[a] In The Analyzed Vegetables

sample	concentration (ng/g)
iceberg lettuce	90 ± 7
romaine lettuce	197 ± 15
escarole lettuce	264 ± 20
lamb´s lettuce	2350 ± 198
kale	710 ± 55
spinach	980 ± 70
cress	955 ± 68

[a] Mean ± standard deviation (n=3).

4

5 **Supplementary material**

6 **Fig. S1.** Effects of the experimental parameters on the extraction efficiency of

7 CPE.

8 **Tables S1 and S2**

9

10

11 **For Table of contents only:**

12

13

14

15