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Analysis of vitamin K in green tea leafs and infusions by SPME–GC-FID

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Abstract

The usual methods for determination vitamin K are laborious. With the aim of replace them by a simpler method that requires fewer solvents, lower analytical levels and allows reporting of results in a smaller period of time, a procedure based on solid-phase microextraction (SPME) and gas chromatography with flame ionization detector (GC-FID) was developed. This method was used to analyse vitamin K in green tea leafs and infusions from nine brands of green tea commercialised in Portugal.

The best analytical conditions were obtained using PDMS 7 μm fibre using immersion extraction at 40 °C, for 45 min and 1300 rpm. The linear range for vitamins K1 and K2 was defined. The detection limits obtained with PDMS fibre and GC/FID were 0.16 and 0.07 mg/L for vitamins K1 and K2, respectively.

In all analysed infusions the concentration of vitamin K was lower than the detection limit of the developed method, however, the concentration of vitamin K1 present in tea leafs was between 120 and 625 μg/100 g.

Keywords: Green tea; SPME; Infusion; Vitamin K1; Vitamin K2

1. Introduction

Tea is an infusion of the *Camellia sinensis* leafs. Originating from China today is the most widely consumed beverage in the world, aside water.

The several types of tea can be included in three categories: not fermented (green tea), partially fermented (oolong) and fermented (black tea) (Graham, 1991). Eighty percent of the tea consumed in the world (Europe, North America and North Africa) is black tea, whereas green tea is drunk throughout Asia and North Africa (Morocco). Epidemiologic observations and laboratory studies have indicated that green tea consumption reduce the risk of a variety of illness, including cancer and showed a preventive effect against atherosclerosis, coronary heart disease and high blood cholesterol concentration (Chung, Schwartz, Herzog, & Yang, 2003; MacKay & Blumberg, 2000; Tijburg, Mattern, Folts, Weisgerber, & Katan, 1997). Due to these facts the popularity of this beverage was grow all over the world.

The tea presents many groups of compounds, like polyphenols, alkaloids, amino acids, glycides, proteins, volatile compounds, minerals and trace elements (Stagg & Millin, 1975). The major group of substances studied in green tea are the antioxidant compounds like catechins (Higdon & Frei, 2003; Wiseman, Balentine, & Frei, 1997). But there are other compounds with interest for human health like fluoride, caffeine, vitamins and minerals, e.g. manganese and chromium (Cabrera, Gimenez, & Lopez, 2003; Powell, Burden, & Thompson, 1998; Xie, von Bohlen, Klockenkämper, Jian, & Günther, 1998).

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One of these compounds, vitamin K, has been implicated in drug-nutrient interactions with warfarin. In 1999 was reported a case of dietary phylloquinone/anticoagulation medication interaction in a patient that consumed green tea. The inhibitor effect was explained by the vitamin K1 present in the infusion (Booth & Centurelli, 1999; Taylor & Wilt, 1999).

Vitamin K has been historically identified for its role in the synthesis of four clotting proteins (factors II, VII, IX and X) and it was found that this vitamin act as an essential cofactor in the post-translational synthesis of γ-carboxyglutamic acid (gla) in theses proteins. Vitamin K-dependent proteins have been identified in the bone, spleen, skin and other organs (Booth, Sadowski, Weihrauch, & Ferland, 1993; Booth & Centurelli, 1999; Burtis, Ashwood, & Tietz, 1994).

Vitamin K exists naturally in two forms: as phylloquinone (vitamin K_1) and menaquinonones (vitamin K_2). The chemical structure of phylloquinone (Fig. 1A) is 2-methyl-3-phytyl-1,4-naphthoquinone having a phytyl group with one double bond as a side chain. It is synthesised by plants whereas menaquinones, 2-methyl-3-(prenyl)$_n$-1,4-naphthoquinone (MK-*n*, Fig. 1B), are of microbiological origin. They are named according to the number of prenyl groups (up to 13) in the unsaturated side-chain. Menadione, formerly know as vitamin K_3, is a synthetic form; its structure is 2-methyl-1-4-naphthoquinone (Fig. 1C). Phylloquinone exists naturally only in the *trans* form, and the all-trans configuration is also the most common one for menaquinones. *Cis-trans* isomers, which are formed during UV light exposure or synthetic production of vitamin K, are considered to have low bioactivity (Booth et al., 1993; Burtis et al., 1994).

Green leafy vegetables contain the highest content in phylloquinone and contribute 40–50% of total intake (Booth & Suttie, 1998).

The first results of vitamin K content in foods were obtained by the chick bioassays (Booth et al., 1993). There were numerous variables inherent in the chick bioassay that affected the final estimates of vitamin K1 in foods. Subsequent analytical methods used for vitamin K1 analysis included thin-layer chromatography and gas chromatography (Booth et al., 1993; Manes, Fluckiger, & Schneider, 1972; Seifert, 1979). The application of high-performance liquid chromatography (HPLC) to the determination of phylloquinone at physiological concentrations in plasma has facilitated the development of more sensitive and precise methods for routine analysis in food but some of these methods are limited to human and cow milk, infant formulas and leafy green vegetables (Booth et al., 1993; Booth, Sadowski, & Pennington, 1995). The HPLC method is associated with several pre-treatment methods to isolate, purify and concentrate the vitamin K from sample matrices. Most of these pre-concentration techniques included liquid–liquid extraction (LLE) and solid phase extraction (SPE). These chromatographic methods also include a derivatization step, in order to improve the detection signal and the resolution between peaks.

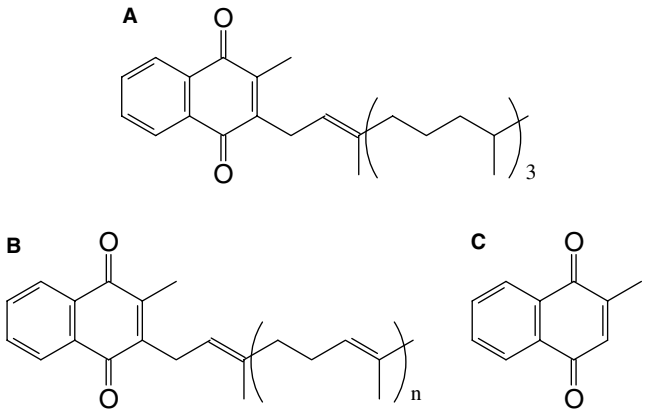

Fig. 1. Chemical structures of phylloquinone (A), menaquinone (B) and menadione (C).

In the last decade the phylloquinone content of several foods has been analysed by HPLC (Bolton-Smith, Price, Fenton, Harrington, & Shearer, 2000; Dismore, Haytowitz, Gebhardt, Peterson, & Booth, 2003; Majchrzak & Elmadfa, 2001; Ostermeyer & Schmidt, 2001; Piironen & Koivu, 2000; Weizmann et al., 2004).

In food samples, a pre-treatment is often necessary to isolate the components of interest from sample matrices, to purify and concentrate the analytes. The solid-phase microextraction is a very convenient technique used for the analysis of volatiles and semivolatiles compounds in liquid samples and nowadays is one of the techniques used in food analysis. SPME requires no solvents or complicated apparatus. The theory of SPME both in immersion and or in headspace technique (HS) has been described in detail by Zhang and Pawliszyn (Pawliszyn, 1997, 1999).

With the aim of replacing the laborious methods by a simpler method that requires fewer solvents, lower analytical levels and allows reporting of results in a smaller period of time, a procedure based on solid-phase microextraction (SPME) and gas chromatography with flame ionization detector (GC-FID) was developed.

For analyse vitamin K in green tea samples several extraction parameters were optimized: type of fibre, extraction type, salt concentration, stirring speed and time of exposure.

Linearity, detection and quantification limits and precision were determined accordingly the requirements of ISO 8466 (ISO 8466-1:1990).

The validated method was used to analyse vitamin K in green tea leafs and infusions from different brands of green tea market in Portugal.

2. Materials and methods

2.1. Apparatus

The chromatographic analysis was performed using a Hewlett–Packard, HP 6890 series GC System coupled to a flame ionisation detector (Agilent technologies, Willington, USA). The gas chromatograph was equipped with a

split-splitless injector, operating in the splitless mode. A capillary column 30 m length, 0.25 mm i.d., 0.25 μm film thickness DB-5 column (5% phenylmethylpolysiloxane, J&W Scientific, Folsom, CA).

A fibre holder for manual use was purchased from Supelco (Bellefonte, PA, USA). Microextraction fibres from Supelco coated with four different films: polydimethylsiloxane (PDMS) 7 μm, polyacrylate (PA) 85 μm, polydimethylsiloxane/divynilbenzene (PDMS/DVB) and polydimethylsiloxane/divynilbenzene/carboxen (PDMS/DVB/CAR). All fibres were conditioned in the hot injector of the gas chromatograph according to instructions provided by the supplier.

The stirring and heating of aqueous solutions and teas were performed using a hot/stirring plate, Bibby Sterilin CD 162 (Staffordshire, UK) with stirring bars 13 mm × 3 mm from Azlon (Bibby Sterilin, Staffordshire, UK).

For chromatographic analysis glass vials taking 20 mm crimp seals from Supelco (20 and 50 mL) were used. Vials were fitted with crimped aluminium caps lined with PTFE-coated butyl rubber septa.

For extraction of vitamin K from leafs was used an ultra sound bath (Selecta, Barcelona, Spain).

For vitamin K determination, the extracts of green tea leafs were dried in a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4001, Germany).

2.2. Reagents and standard solutions

All the aqueous solutions and serial dilutions were prepared by dilution with bidistilled-deionized water (Mili-Q System, Millipore, Billerica, MA, USA).

The sodium chloride, sodium sulphate, methanol, isopropanol, hexane and silica gel 60 (0.2–0.5 mm), *pro-analysis* grade, were supplied by Merck (Darmstadt, Germany).

The standard of vitamins K1 and K2 were supplied by Sigma (Sigma–Aldrich, St. Louis, MO). The stock solutions and serial dilutions were prepared with methanol.

These solutions were stored at 4 °C in the absence of light. For SPME optimization studies, appropriate amounts of the intermediate standard solution were added to bidistilled-deionized water (Milli-Q system) to obtain final concentrations in the mg/L level. Thirteen solutions containing the two standards with concentrations between 0.08 and 4.4 mg/L were prepared to study linear range and the approximate concentrations were: 0.08, 0.16, 0.24, 0.32, 0.40, 0.80, 1.2, 1.6, 2.0, 2.4, 3.2, 4.0 and 4.4 mg/L.

2.3. Tea samples

Nine samples of green tea of various brands were purchased from several shops in Lisbon (markets and herbalists). Five of them are sold in tea bags and the other three as loose-leaf tea. The different brands of green tea were represented by the acronyms G, S, T, TN, TV, F, B, M and T. All teas were analysed in triplicate.

The time and ratio of green tea leafs and water was kept constant during the study and according the instructions provided by the manufactures. These conditions try to seem similar to those made by all consumers in their houses.

A mass of 1.5 g of tea leaf (or tea leaf from a bag) was weighted in a beaker and 250 mL of boiling bidistilled-deionized water (Milli-Q system) was added. Since the manufactures recommend different times of brewer (6–10 min), we defined 10 min. After this period, the tea infusions were filtered into a new beaker. The analysis of vitamin K was made directly in green tea infusions (SPME–GC-FID method).

2.4. Extraction of vitamin K from green tea leafs

The extraction procedure is similar to that reported by Booth, Sadowski, Madabushi, and Davidson (1995). To 10 g of green tea leafs was added 150 mL of isopropanol: hexane (3:2 v/v) and 50 mL of bidistilled-deionized water (Milli-Q system), followed by a mixing in an ultra sound bath for 30 min. The solution was transferred to a conical flask and added 100 mL of bidistilled-deionized water (Milli-Q system). After mixing, two layers were forms. The hexane layer was removed and the aqueous layer was washed twice with 25 mL of hexane. All the hexane layers were pooled together and 15 g of anhydrous sodium sulphate was added to hexane extract to remove any residual water. The sample was filtrated and the extract was evaporated to dryness on a rotary evaporator. The residue was dissolved in a 10 mL hexane. Six grams of silica gel was added to this hexane extract and was filtrated and evaporated to dryness in a water bath. The residue obtained was dissolved in 50 μL of methanol and reconstituted in 25 mL of 10% NaCl solution, followed by the SPME–GC-FID procedure.

2.5. SPME–GC-FID method

The chromatographic conditions were previously optimised and validated for the analysis of vitamin K (Reto, Figueira, & Almeida, 2003). The detector and injector temperatures were set at 325 and 250 °C, respectively. The splitless time was 5 min. A desorption time of 5 min at 250 °C was enough for a quantitative desorption of vitamin K as the reinsertion of the fibre after the run did not show any carry over. The carrier gas was helium at 1 mL/min and, for FID, air at 450 mL/min, hydrogen at 45 mL/min and nitrogen at 20 mL/min were used. The following temperature program was used: 90–300 °C at a rate of 10 °C/min and 13 min at 300 °C.

On the beginning of each working day, a column blank was followed by a fibre blank and reagent water blank (bidistilled-deionized water, Milli-Q system) to detect any possible laboratory contamination.

In order to optimise the best conditions to analyse vitamins K1 and K2 by SPME we studied several factors that

can affect SPME efficiency like fibre coating film, temperature, extraction time, salt effects and stirring. All solutions were analysed in triplicate ($N = 3$). The best results were obtained with 25 mL of infusion, PDMS 7 μm fibre, 45 min, 10% NaCl, 40 °C and 1300 rpm.

To an aliquot of 25 mL of sample (infusions or leafs) was added 2.5 g of sodium chloride. After placing a stir bar in each vial, it was sealed with an aluminium seal with a PTFE septum. The vials were placed on a hot/stirring plate at 40 °C and 1300 rpm. In order to monitor temperature, an external probe was placed in a vial containing bidistilled-deionized water (Milli-Q system) under the same conditions and in parallel on the same hot/stirring plate. The samples were heated for 30 min. For extraction, the fibre was pushed out and exposed by immersion in the solution for 45 min (the stainless steel needle was kept 2 cm below the septum). After extraction, the fibre was immediately inserted into to GC injector for desorption. A desorption time of 5 min at 250 °C was enough for desorption of all analytes from green tea infusions and leafs.

3. Validation studies

The linear range was studied by analysis of 13 solutions containing the standards in different concentration levels (0.08–4.4 mg/L – preliminary working range). The study of linearity included the statistical linearity test with the determination of the test value PG required for the *F*-test (ISO 8466-1:1990).

Ten solutions containing the target compound at the lowest concentration of linearity range were analysed and the standard deviations (SD) were determined based on the areas obtained for each compound. The values of limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formula ($3 \times SD$) and ($10 \times SD$), respectively.

The linear ranges, the PG values, *F* value of Fisher/Snedecor (tabled value), LOD and LOQ for the vitamins are given in Table 1.

The repeatability of the optimised direct immersion SPME–GC-FID technique was studied by analysing 10 replicate samples of bidistilled-deionized water (Milli-Q system) spiked with vitamin K1 (0.18 mg/L) and vitamin K2 (0.14 mg/L).

4. Results and discussion

Fig. 2 presents the chromatogram of a solution containing the vitamins K1 and K2 with the corresponding retention time.

4.1. SPME fibre selection

Some preliminary tests were used to find out the most suitable fibre type for vitamin K analysis. Non-polar compounds are best extracted with a polydimethylsiloxane fibre (PDMS) and polar compounds with the polyacrylate fibre (PA). Accordingly to the chemical structure of vitamin K,

Table 1
Regression data for vitamins K1 and K2 by optimised SPME–GC-FID and their detection and quantification limits

Compound	Linearity range (mg/L)	R^2	*N*	*m*	*b*	CV (%)	PG	*F*	LOD (mg/L)	LOQ (mg/L)
Vitamin K1	0.24–2.0	0.992	7	911	−243	7.1	4.8	7.7	0.16	0.54
Vitamin K2	0.16–4.0	0.992	9	553	−168	9.8	2.3	5.9	0.07	0.23

Legend key: R^2 – square correlation coefficient; *N* – number of concentration levels; *m* – slope; *b* – intercept; CV (%) – coefficient of variation of the method; PG – test value; *F* – tabled value of Fisher/Snedecor (95% confidence level); LOD – limit of detection; LOQ – limit of quantification.

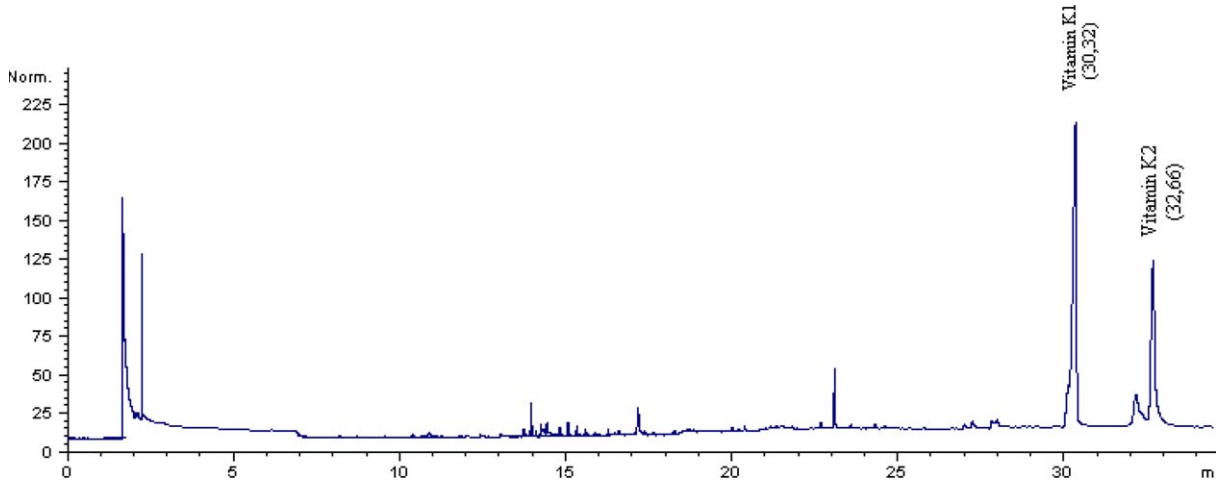

Fig. 2. Chromatogram of vitamins K1 and K2 by SPME–GC-FID (immersion, 25 mL, 45 min, 10% NaCl). Programme: 90–300 °C at a rate of 10 °C/min and 13 min at 300 °C.

the fibre more suitable for the analyse of vitamin K is the PDMS fibre with a thin film (7 μm) although we tested four different fibres.

Fig. 3 shows the areas obtained for vitamin K by SPME–GC-FID extraction with three different fibres. The analyte concentration (0.5 μg/L), the sample volume (25 mL) and the extraction conditions (45 °C, 1300 rpm, and 40 min) were the same in all cases.

As could be expected, the best results were obtained with the PDMS 7 μm fibre and all subsequent experiments were performed with this fibre coating.

4.2. Salt effect

The addition of salt increases the ionic strength of the solution and the organic compounds become less soluble increasing the partition coefficients between phases, and therefore, their affinity to the fibre coating. The selected salt was NaCl and four different concentrations were tested: 0%, 8% and 10%. The obtained results are represented in Fig. 4.

The salt concentration is an important factor in the analysis of vitamin K by SPME. The areas obtained with 10% of NaCl were higher than those obtained with the other concentrations and therefore all subsequent analysis was made with 10% NaCl.

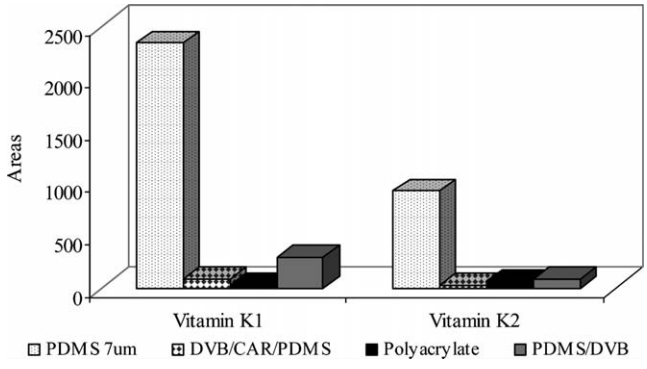

Fig. 3. Comparison of response of SPME analysis with four fibres.

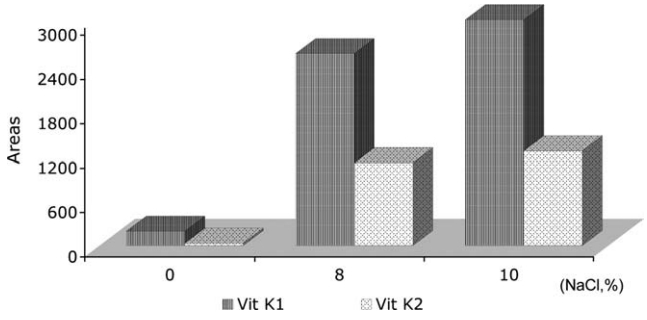

Fig. 4. Comparison of response of SPME analysis with increasing salt concentration.

4.3. Exposure time

The exposure time is very important for several reasons – it influences the partition of solutes between solvent and fibre coating, and this has direct influence on the analysis time and performance. Five different exposure times were tested, in order to determine the best compromise between time and analyte response, and establish extraction profiles of the vitamin K (Fig. 5).

The areas of vitamin K increase with the increase of exposure time but there are no significant differences between the results obtained for the two highest extraction times, 45 and 60 min (P-values > 0.05 at the 95% confidence level). Therefore, an exposure time of 45 min looked suitable and convenient for a chromatographic run of 35 min.

4.4. Effect of the temperature

Comparing the average areas at different temperatures, differences in areas are observed for vitamins K1 and K2 (Fig. 6). If analysis of variance (ANOVA) is applied to these results a P-value smaller than 0.05 is obtained at the 95% confidence level. Therefore, the extraction temperature is significant for these compounds.

The most efficient extractions were obtained at 40 and 60 °C but there are no significant differences between these two temperatures. Therefore a temperature of 40 °C was chosen because higher temperatures can decrease the partition coefficients between the fibre coating and the sample.

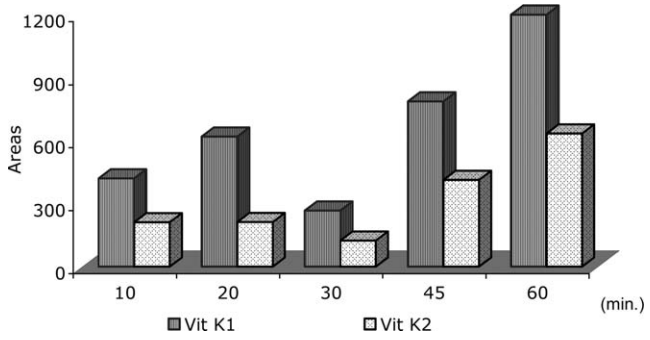

Fig. 5. Extraction profiles of the vitamins K1 and K2.

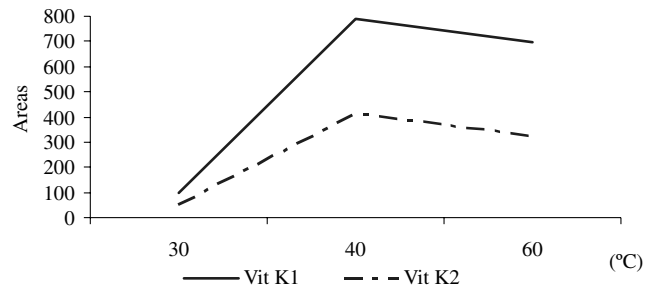

Fig. 6. Influence of the temperature in extraction by SPME with PDMS 7 μm coated fibre.

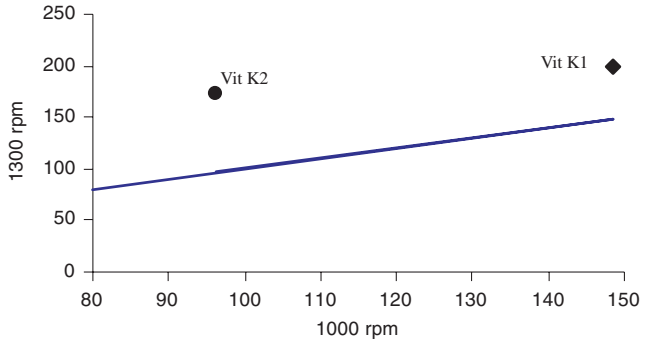

Fig. 7. Comparison of extraction of vitamins K1 and K2 between 1000 and 1300 rpm by immersion SPME with PDMS 7 μm fibre.

4.5. Stirring effect

Stirring is one parameter that can decrease equilibration time when SPME sampling is performed by direct immersion. For vitamin analysis, we tested static SPME (0 rpm), 1000 and 1300 rpm. There were significant differences between static and stirred samples by SPME. Without stirring, the vitamin K1 was not extracted and therefore we were compared the obtained results between 1000 and 1300 rpm (Fig. 7).

The best results were obtained at 1300 rpm and therefore all subsequent analysis was made with this stirring speed.

Taking into account these previous results, the best conditions of analysis the vitamin K by SPME were: PDMS 7 μm fibre, 10% NaCl, 40 °C extraction temperature, 45 min of extraction time and 1300 rpm.

The method shows good square correlation coefficients (R^2) and is linear in study range (PG < F).

The repeatability of the method is expressed as relative standard deviation (RSD) and was 8.4% and 4.7% for vitamins K1 and K2, respectively. For the purpose of this method it may be considered acceptable a RSD of 10% or less, therefore the precision of this method is considered good.

4.6. Green tea infusions

Tea was been listed as a good source of vitamin K but authors have reported negligible amounts in the infusions. Booth et al. detected this vitamin by reverse phase HPLC and the content on tea brews was 0.3 μg/L (Booth et al., 1995). Comparing this value and the LOD by SPME–GC-FID we concluded that it was not possible to detect vitamin K in the infusions. However, to confirm this fact the nine samples of green tea were analysed.

Since it was not possible to detect vitamin K directly from the tea infusions was decided to quantify this vitamin in leafs. The extraction of vitamin K from leafs was made by sonication of the leafs with organic solvents. The vitamin K2 was not detect in any green tea samples (the concentration is lower than the limit of detection). The vitamin K2 is synthesised by microorganisms and their content in leafs must be lower than the vitamin K1. Table 2 shows the obtained results for vitamin K1.

Table 2
Content of vitamin K1 in green tea leafs by SPME–GC-FID

	Vitamin K1 (μg/100 g)
TV	202
TN	200
T	215
D	195
F	262
M	182
S	120
G	625

The content of vitamin K1 in green tea leafs was between 120 and 625 μg/100 g. These differences between green tea brands were significant ($p < 0.05$) but these differences were also detected by Booth et al. (1995). However values obtained in this study were lower than those obtained by Booth et al. (1993), Booth et al. (1995). The fact that the samples were from different brands can explain the variability observed (different geographic point of origin, agricultural practices, storage and methods of processing).

Vitamin K is a fat-soluble vitamin and their extraction with boiling water during the infusion it is not very effective. For this reason the content of vitamin K found in infusions (0.3 μg/L) (Booth et al., 1993) is lower than those found in green tea leafs.

Biological, chemical and chromatographic methods have been used to determine vitamin K in foods. The gas chromatography was used during 1960 and 1970 for the vitamin K analysis. But the obtained retentions times were long and detection limits were high. However after the introduction of new methodologies like SPE and SPME, this method has again become object of interest. Solid-phase microextraction provides many advantages over conventional sample preparation techniques. The SPME method is simple, rapid, solventless, economical and versatile. The extraction, pre-concentration and analyse are combined in a single step. Based on these facts a new method SPME-GC-FID was developed for the analysis of vitamin K, one of the compounds in green tea infusions related with adverse effects. The optimised method is not suitable for analysis of vitamin K in infusions because the content of this vitamin in infusion is much lower than the detection limit of the method. However the procedure is suitable for vitamin K analysis in leafs. The SPME–GC-FID method showed a good linearity ($R^2 \cong 0.99$; PG < F), repeatability (RSD < 10%) and the limit of detection is 0.16 and 0.07 mg/L for vitamins K1 and K2, respectively.

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