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Article

Factorial Experimental Design Optimization of Solid Phase Microextraction (SPME) Conditions for Analysis of Butylated Hydroxytoluene (BHT) in Bottled Water

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BHT é um antioxidante usado como aditivo em alguns alimentos e em plásticos de embalagens, e sua presença em água mineral engarrafada é possível devido à migração do aditivo contido nas paredes do recipiente. Planejamento fatorial foi empregado para determinar os valores ótimos para os parâmetros operacionais principais na análise de hidroxitolueno butilado (BHT) em água engarrafada usando microextração em fase sólida (SPME) direta e em *headspace* na etapa de pré-concentração. Os parâmetros otimizados foram pH, temperatura e força iônica da amostra. A separação, detecção e quantificação dos extratos foi efetuada por cromatografia gasosa acoplada a espectrometria de massas. Experimentos preliminares foram realizados para selecionar a melhor fibra para o procedimento de extração. Fibras de polidimethilsiloxano (PDMS) foram selecionadas, e a metodologia otimizada aplicada com sucesso na extração e quantificação de BHT em amostras reais de água mineral e mineralizada engarrafada.

BHT is an antioxidant utilized as additive in some foods and in packaging plastics, and its presence in bottled mineral water is possible due to its migration from bottle walls to the contents. A factorial experimental design was utilized to obtain the optimum values for the main operational parameters in the analysis of butylated hydroxytoluene (BHT) in bottled water using headspace and direct solid-phase microextraction (SPME) in the pre-concentration step. The parameters optimized were sample pH, temperature and ionic strength. The separation, detection and quantitation of the extracts were performed by gas chromatography coupled to mass spectrometry. Preliminary experiments were made to select the best fiber for the extraction procedure. Polydimethylsiloxane (PDMS) fibers were selected, and the optimized methodology was successfully applied to the extraction and quantitation of BHT in real samples of mineral and mineralized bottled water.

Keywords: SPME, GC-MS, factorial experimental design, BHT, bottled water

Introduction

Solid Phase Microextraction, introduced by Arthur and Pawliszyn in 1990 [1] as a modern alternative to traditional sample preparation technology, is able to address many of the requirements put forward for analytical research.[2] Several advantages can be pointed out in relation to this technique, such as it is solvent free, fast, uses the whole sample for analysis, requires only small amounts of sample and the fibers are highly reusable. It has been successfully applied for the analysis of diverse organic compounds from different matrixes.[3]

Application of SPME to determinate phenolic compounds has already been reported.[4] The European Union (EU) has classified several phenols as priority contaminants and the 80/778/EC directive states a maximum concentration of 0.5 μg L^{-1} for total phenols in drinking water (individual concentrations should be under 0.1μg L^{-1}).[5] Since 1947, 2,6-di-*tert*-butyl-4-methylphenol or 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) is an antioxidant widely used especially as an additive in some industrialized foods and packaging plastics[6] and it may thus migrate into the packages contents.[7] Controversial effects of BHT ingestion on public health were considered.[8] A preliminary report on the specific application of direct immersion SPME for BHT determination (using a PDMS-coated fibers) in plastic-bottled water was already done,[9] suggesting that this technique is a good alternative for fast and accurate analyse; however, further optimization optimization was found to be necessary before proposing it as a routine procedure.

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SPME method optimization can be achieved in a traditional univariate trial, studying each factor separately, or by a chemometric approach based on the use of an optimum set of experiments (experimental design) which allows the simultaneous variation of all experimental factors studied, and the distinguishing of interactions among them that are not detectable with the classical experimental methods.[10]

This paper deals with the optimization of the analysis of BHT in water samples using such an experimental design. After preliminary, univariate studies evaluating the capabilities of different fibers, the effects of temperature, pH and salinity were evaluated by a two-level factorial design to obtain the optimal conditions for direct immersion (D-SPME) and headspace (HS-SPME) modes. Finally the performance of the optimized method was demonstrated analyzing real samples of mineral and mineralized bottled water.

Experimental

Equipments

All chromatographic separations were performed using a HP-6890 GC coupled to a HP-5972 Mass Selective Detector (Hewlett-Packard Corp., Avondale – PA) fitted with a HP-5 capillary column (30 m x 0.25 mm x 0.25 μm). Helium at 1 mL min^{-1} (36 cm s^{-1}) was used as a carrier gas. The following column oven temperature program was adopted: 80 °C to 210 °C at 20 °C min^{-1} and then hold for 1 min. All injections were performed manually. In all cases, the SPME fiber was kept for 5 min in the injection port at 250 °C operated in splitless mode; under these conditions, no peaks were observed in blank runs performed between injections. The MS was operated with an electron energy of 70 eV. For the preliminary experiments and for the SPME method optimization, runs were performed in the TIC mode (scanned mass range form *m/z* = 35 D to 500 D); for quantitative experiments SIM mode was adopted, monitoring the fragment with *m/z* = 205 D (peak with a relative abundance of 100% in the BHT spectrum). Peaks were identified by comparison with the mass spectra library of Hewlett-Packard (NBS75K) and with retention data time of known standards.

Chemicals and solutions

BHT (>99% purity) was purchased from Merck-Schuchardt, Germany. A primary stock solution (20.00 g L^{-1}) of BHT was prepared by dissolving 0.5000 g of this analyte in 25.00 mL of pesticide-grade acetone (U.V.E., Buenos Aires, Argentina); the working 40 mg L^{-1} stock solution was prepared diluting the primary stock in acetone. Both stock solutions were stored at 4 °C in the refrigerator. Aqueous test samples (renewed each day) were prepared by dilution of suitable amounts of the working stock solution in deionized water. Other chemicals included analytical grade NaCl (Anedra, Buenos Aires, Argentina) and a Titrisol 0.1 mol L^{-1} HCl solution (Merck, Darmstadt, Germany).

SPME preliminary univariated experiments

To select the best fiber for the remaining experiments, fibers coated with 100 μm polydimethylsiloxane (PDMS), 85 μm polyacrylate (PA) and 65 μm polydimethylsiloxane - divinylbenzene (PDMS-DVB) housed in manual SPME holders (Supelco, Bellefonte - PA), were tested. The fibers were conditioned prior to use according to the supplier's instructions. Direct extractions from a 19.8 μg L^{-1} aqueous test solutions at pH 6.5 and room temperature, without addition of NaCl and with extraction times ranging from 15 min to 60 min, were tested with the three fibers. Unless stated to the contrary, all measurements were done in triplicate.

Multivariated optimization of extraction conditions

The optimization of the main operational parameters of the SPME procedure (sample pH, temperature and ionic strength) was carried out both for direct (D-SPME) and headspace (HS-SPME) modes, using a 40 μg L^{-1} aqueous solution of BHT as test sample. Direct SPME was performed by placing 15 mL sample aliquots in amber vials (maximum capacity) capped with PTFE-coated septa; HS-SPME was performed by placing 10 mL aliquots in similar vials. The extraction time was 15 min. A multivariate simultaneous approach was initially adopted to study the effects of variations of the chosen parameters on extraction efficiency, using a set of experiments arranged through a 2^3 factorial design.[11] The values for the upper (+) and lower (-) levels of each variable were: pH 2.5 and 6.5; temperature = 25 ºC and 75 ºC; and ionic strength = zero and 30 g NaCl/100 mL solution. Special statistical treatment was performed using the trial version of the software Design-Expert 6.0.7 (Stat-Ease Inc., Minneapolis - MN). To complement this multivariate assessment, univariate studies of ionic strength (using aqueous samples containing zero to 30% NaCl w/v) and extraction times (from 5 min to 60 min) were also performed.

Quantitative evaluation

To determine the linearity and limits of detection of the optimized method, an analytical curve was determined

using aqueous solutions of BHT with concentractions ranging from 0.04 μg L^{-1} to 40.0 μg L^{-1}. For each point triplicate extractions were carried out. Then real samples of commercial and mineralized bottled water were analyzed using external standard calibration.

Results and Discussion

Fiber screening

Figure 1 shows the extraction profiles (peak area × extraction time) for the fibers evaluated. It is clear that, for all fibers, the equilibration time is greater than 60 min. For this extraction time, the fiber extraction efficiency order is PA < PDMS/DVB < PDMS. To determine the statistical significance of the differences observed between the fibers, the data was submitted to an ANOVA test complemented by the *post hoc* contrast test of Scheffé,[12] whose results are shown on Table 1. The results of the ANOVA test shows that, with 95% probability and except for short extractions (t_{ext} = 15 min), the results obtained with the tested fibers can be considered statistically different ($F > F_{crit}$). A more detailed insight can be provided by the Scheffé test: the efficiency of PA fiber is different from that of the other fibers for extraction times greater than 15 min; for 60 min extractions, all efficiencies are different. Considering the higher extraction efficiency provided, 100 μm PDMS was selected for the remaining experiments.

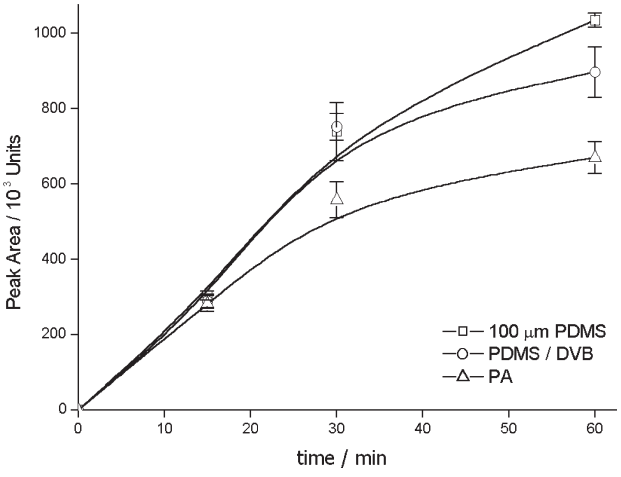

Figure 1. BHT extraction profiles for 100 μm PDMS, PDMS / DVB and PA fibers. Direct extractions from 19.8 μg L^{-1} neutral aqueous solutions of BHT.

Table 1. Results of ANOVA test (F-values and associated probabilities, P) between the PDMS, PDMS / DVB and PA peak areas for 15 min, 30 min and 60 min extraction, and corresponding Scheffé contrast significance tests

t/min	F [a]	P%	Scheffé test
15	1.21	36.3	all fibers are equivalent
30	10.9	1.00	PA ¹ PDMS and PA ¹ DVB
60	46.3	0.02	no equivalence between fibers

[a] F_{crit} (2,6) = 5.14 (95% confidence level).

Multivariated optimization of the SPME method

Figures 2 and 3 shows the peak areas found for BHT after direct and headspace extractions using PDMS 100 mm fiber and different operational conditions. The effects of the studied variables on the extraction efficiency and the corresponding interactions between the variables calculated after the 2^3 factorial planning experiments, as well as the ANOVA analysis of these effects, to assess their statistical significance, are shown in Table 2.

Both for direct and headspace SPME, the most important operational variables are the temperature and ionic strength of the sample. According to Table 2, the temperature accounts for 22.1% (D-SPME) and 32.6% (HS-

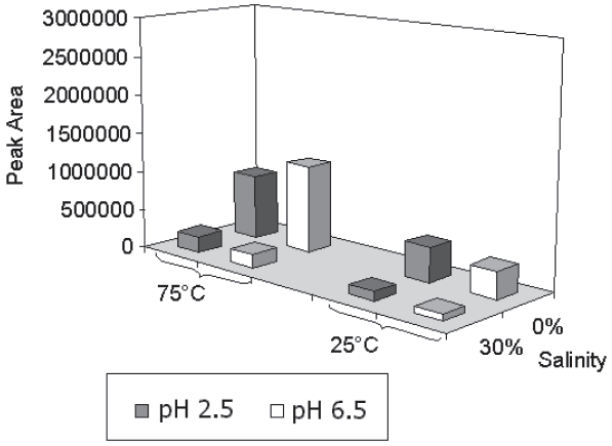

Figure 2. Peak areas for D-SPME of 40 μg L^{-1} aqueous solutions of BHT obtained under varied operational conditions.

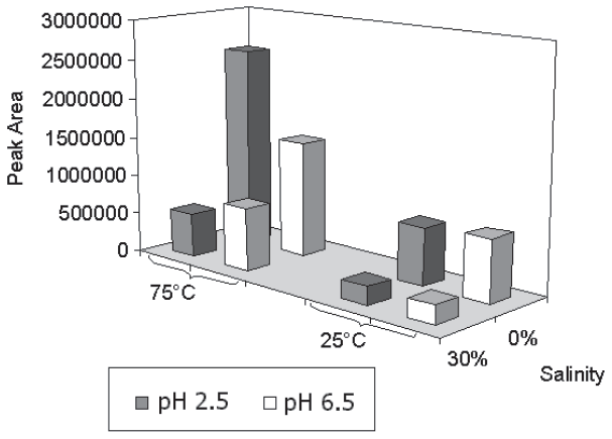

Figure 3. Peak areas for HS-SPME of 40 μg L^{-1} aqueous solutions of BHT obtained under varied operational conditions.

Table 2. Effects and interactions from the 2^3 factorial design experiments for direct (D-) and headspace (HS-) SPME of BHT, their relative percent contribution to the total variance (Contr.%), and significance from ANOVA test F-values and associated probabilities, P

		Factorial Design		ANOVA	
	Variable	Effect/10^3	Contr.%	F	P%
D-SPME	T	338	22.1	84.6	< 0.01
	i	-541	56.8	217.18	< 0.01
	pH	30	0.18	0.68	42.3
	T × i	-248	12.0	45.70	< 0.01
	T × pH	97	1.82	6.96	1.8
	i × pH	-73	1.05	4.00	6.3
	T × i × pH	-98	1.89	7.22	1.6
HS-SPME	T	896	32.6	190.96	< 0.01
	i	-988	44.4	227.13	< 0.01
	pH	-193	1.66	10.36	0.67
	T × i	-454	8.96	42.81	< 0.01
	T × pH	-204	1.54	13.40	0.29
	i × pH	330	4.85	26.67	0.02
	T × i × pH	332	4.92	30.23	0.01

T: temperature; i: ionic strength.

SPME) of the total observed variance; as for ionic strength, the contribution of this parameter is, respectively, 56.8% (D-SPME) and 44.4% (HS-SPME). Both for D- and HS-SPME, the temperature effect is positive and the ionic strength effect is negative (*i.e.*, increasing the temperature and decreasing the ionic strength of the media would result in better extraction efficiency). Regarding the temperature effect, for direct extraction its impact on the extraction equilibrium depends on the variation of the enthalpy associated to the partition of the analyte between the fiber coating and the sample: the observed reduction of the extracted amounts of BHT with higher extraction temperatures shows that this process is endothermic,[13] which is not usual in SPME. As for HS-SPME, the temperature/efficiency dependence is more complex[14] and, therefore, such a direct interpretation of the observed temperature effect is not possible.

The effect of ionic strength on BHT extraction also is not that customarily found in direct SPME (normally, addition of NaCl to the sample improves the extraction efficiency both for direct and headspace operation). However, phenols such as BHT contain a hydroxyl group in their structures, which can increase their affinity for the solutions containing electrolytes and result in the observed comportment, which has already been reported for the extraction of related chemical species.[15] Anyway, considering that in the multivariate experiments only two levels of this variable were tested, a further confirmation of this odd observation was performed with headspace extractions of samples with NaCl concentrations ranging from 3.75% to 30%, using T = 75 ºC and pH = 2.5. The results of this additional study are shown in Figure 4. It can be clearly seen that there is a sharp, roughly linear decrease on the extraction efficiency with the concentration of NaCl (a 1.0% increment on the concentration of NaCl causes a reduction of ~2.7% in the BHT peak area), which confirms the results from the factorial design.

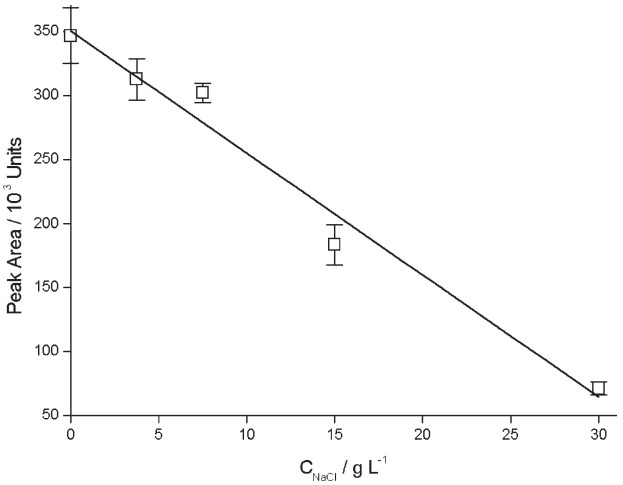

Figure 4. Dependence between BHT peak areas and concentration of NaCl on the sample after HS-SPME.

Regarding the effect of pH and adopting 95% as the confidence level, its impact is not statistically significant for D-SPME. For HS-SPME its effect is significant, although its contribution to the total variance (1.66%) is much less pronounced than that of the other variables. Phenols - such as BHT - are weak acids and control of the pH of the sample is in general considered mandatory in procedures involving these species, potentially having a pronounced impact on method sensitivity.[16] However, since BHT is expected to be a weaker acid than other phenols due to the bulky alkyl groups present in its molecule,[17] the effect of pH on its extraction is expected to be marginal, specially for the low pH values adopted of this study (2.5 and 6.5), an this agrees with the data obtained. Finally, the negative pH effect on HS-SPME is in agreement with general SPME theory: lowering the sample pH shifts the BHT dissociation equilibrium towards the formation of the neutral extractable species, improving the extraction efficiency.

Another aspect of Table 2 worthy if discussing is the interaction between the variables (e.g., **T × i**): for both extraction modes, all interactions (except **i × pH** for D-SPME) are statistically significant. The implication of this observation is that conventional univariate methods can not be used to optimize the operational conditions studied here. Therefore, experiments planned according to multivariate procedures, such as the factorial design employed here, should be used since the impact of these variables upon the extraction efficiency is interdependent.

Several simultaneous physico-chemical processes occurs during the extraction: transport of the analyte through the bulk matrix to the fiber/sample or fiber/headspace interface, its diffusion through the headspace, the static layer surrounding the fiber and the PDMS fiber coating, acid dissociation, *etc.*[14] It is reasonable to suppose that the combined effects of the studied media-conditioning variables on the kinetics and thermodynamics of the involved unitary process result in a complex overall effect, not predictable or able to be determined by a simple univariated study. It is also noted that, for HS-SPME (which, compared to D-SPME, involves additional mass transfer steps), the impact of the measured inter-variable interactions is even more expressive, as indicated by the corresponding **F** parameters and the associated probabilities: even at a 99.5% confidence level these figures are statistically significant.

The optimum values for the parameters studied on the factorial planning experiments discussed above are T = 75 ºC, no NaCl addition and pH 2.5 (HS-SPME) or 6.5 (D-SPME). Under the optimized conditions the extraction efficiency of HS-SPME is remarkably superior to that of D-SPME. Therefore, this extraction mode was selected for the remaining experiments. The determination of the equilibrium time for HS-SPME under the optimized conditions was performed after extractions of solutions containing 40 μg L^{-1} BHT for times ranging from 5 min to 30 min. The extraction profile obtained is shown in Figure 5. It can be seen that equilibrium is reached after *ca.* 15 min of extraction, which was therefore applied for the remaining experiments.

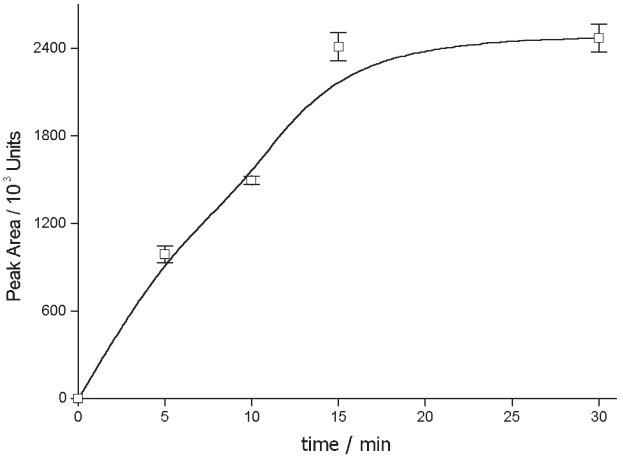

Figure 5. HS-SPME extraction profile for BHT using the optimized procedure.

Quantitative evaluation and application of the HS-SPME method

The quantitative figures of merit of the optimized HS-SPME method (linearity, sensitivity and precision) were assessed through the analytical curve shown in Figure 6. The precision is acceptable: the correlation coefficient of the curve is 0.9999 and the relative standard deviation of the data ranges from 1.3% to 10.1%. Although there is no visible deviation of linearity in the plot, an additional ANOVA test was performed to check for lack of fit in the linear regression.[18-21] Since the estimated **F** value of 0.0777 (**P** = 98,8%) is lower than the $\mathbf{F}_{crit}$ for 95% confidence level ($\mathbf{F}_{crit}$ = 3.71), the null hypothesis (the straight line model describes the relationship between signal and BHT concentration) cannot be rejected. The limits of detection and quantitation (defined respectively as three and ten times the ratio between the standard deviation of the intercept and the slope) were 0.43 μg L^{-1} and 1.45 μg L^{-1}.

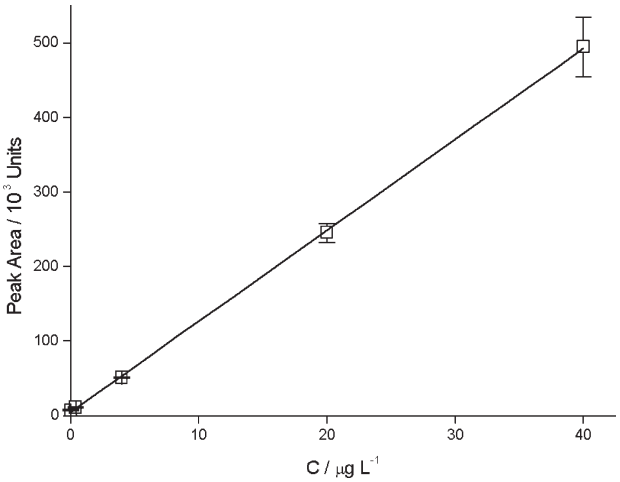

Figure 6. External standardization HS-SPME analytical curve for 0.04 μg L^{-1} to 40 μg L^{-1} BHT. Curve equation: C = (12.20 ± 0.09) A + (4.6 ± 1.8); C = μg L^{-1} BHT and A = 10^3 area units.

To demonstrate the performance of the optimized SPME method, nine mineral and mineralized bottled water samples were analyzed for the presence of BHT. The results are shown in Table 3. The contaminant was found only in three out of the nine samples studied; in the samples where BHT was detected, its concentration was on the μg L^{-1} level.

Table 3. Concentrations of BHT (μg L^{-1}) found in samples of commercial mineral and mineralized bottled water (average of triplicate measurements)

Sample	V / L [a]	C
A	0.5	2.3 ± 0.4
A	1.5	2.1 ± 0.2
B	0.5	n.d. [b]
C	0.5	n.d.
D	1.5	n.d.
E	0.5	1.5 ± 0.1
F	1.5	n.d.
G	2.0	n.d.
H	0.35	n.d.

[a] Volume of the bottle; [b] n.d. = not detected.

Conclusions

A two-level factorial experimental design enables obtaining optimal conditions for Solid Phase Microextraction of BHT in water with a reasonable number of experiments and taking into account the interaction between the variables affecting the SPME process. Extraction times were reduced to half and the limits of detection and quantification lowered an order of magnitude, compared to previous preliminary work.[9] This optimized methodology was successfully applied to the extraction of BHT in real samples of mineral and mineralized bottled water. BHT was detected in one out of three of the samples, and the measured concentrations were above the recommended levels according to the European Union standards for total phenols in drinking water.[5]

Acknowledgements

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