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Gas chromatography/mass spectrometry method for determination and confirmation of BHA, BHT and TBHQ in vegetarian ready to eat meals

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ABSTRACT

Synthetic phenolic antioxidants (SPAs) are permitted in a limited number of food products according to international legislations, with individual maximum limits in each case. This study describes an in-house validated GCMS method for quantitative determination and confirmation for the TBHQ, BHA and BHT in vegetarian ready to eat meals. Develop and optimised simple and rapid sample preparation method for extraction of the three SPAs from the ready to eat food items. Analytical characteristics of the GCMS method such as limit of detection, linear range, and recovery were evaluated. By using external standard method the analytical results showed that the linear correlation coefficients of TBHQ, BHA and BHT were more than 0.998 and Recoveries (n = 6) of the SPAs when spiked to ready to eat food at 5,10 and 30 mg kg^{-1} were in the ranges 97.3-105.2 % for BHA, 102.7-104.7 % for BHT and 98.4- 101.5 % for TBHQ. The lowest detection limit was 0.1 mg kg^{-1} for TBHQ & BHA and 0.05 mg kg^{-1} for BHT. The levels of SPAs in all food items analysed were below the legal limits.

KEYWORDS

Phenolic antioxidants;
BHA;
BHT;
TBHQ;
Extraction methods;
GCMS/MS chromatography;
Food analysis.

INTRODUCTION

The use of synthetic phenolic antioxidants (SPAs) is subject to regulations that establish permitted compounds and their concentration limits. Therefore, a lot of research has been conducted to determine the presence and quantities synthetic phenolic antioxidants (SPAs) in different oil & foods item. According to the recent survey, the Indian food market show, the amount of money Indians spend on meals outside the home has more than doubled in the past decade, and is expected to double again in about half that time. The rapid economic development, innovative technology and food production, growing consumerism and improved lifestyle are the main reasons behind this growth .Value addition of food products is expected to increase from the current 8 per cent to 35 per cent by the end of 2025. The market for semi-processed/cooked and ready to eat foods was around Rs 82.9 billion in 2004-05 and is rising rapidly. Ready-to-eat foods are now greatly preferred by the public; in this development, ready to eat meals play a major role. Ready to eat meals invariably contain fats and oils, which can oxidize slowly during storage. Various oxidation products cause rancidity and

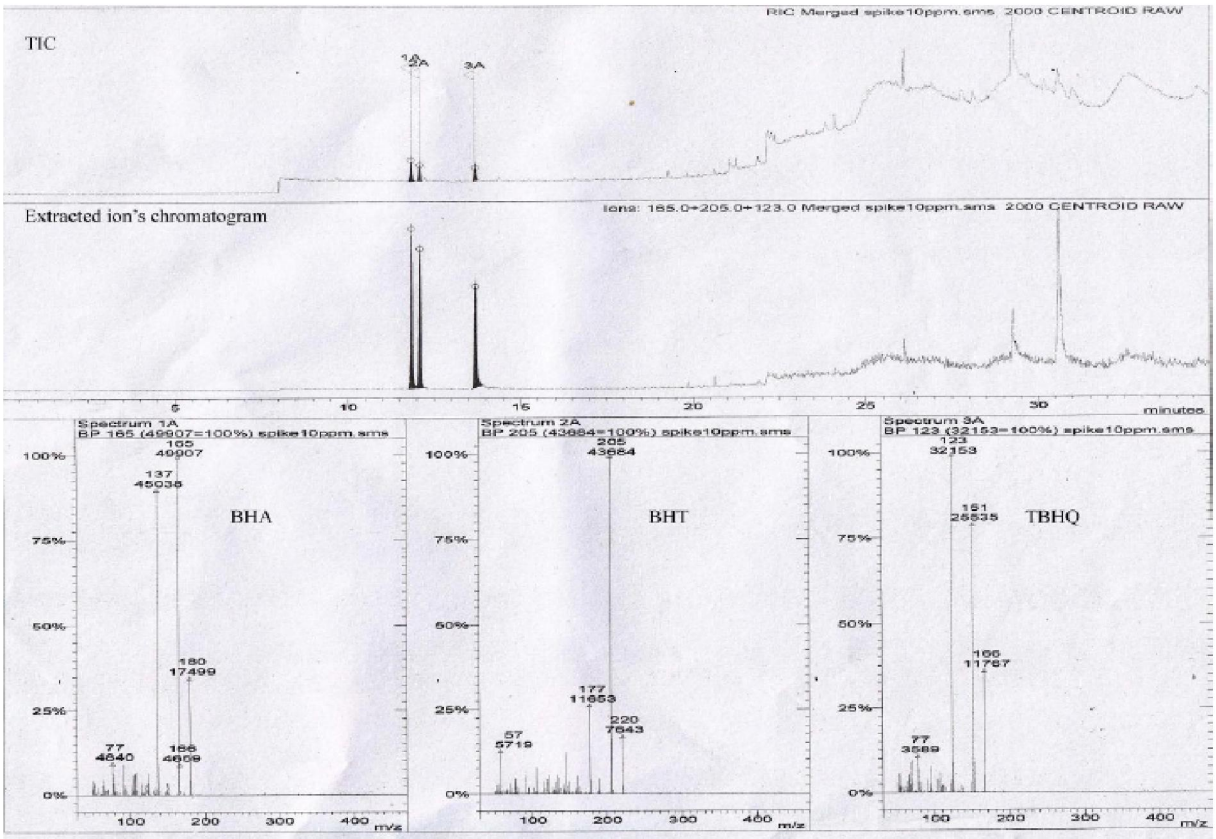

Figure 1 : GC/MS chromatogram (TIC & Extract ion's chromatogram) and mass spectra of three antioxidants. The three peaks showed the following fragment ions (m/z; numbers in parentheses were used for conformation) 1A:- BHA, 165, 137,180,166 2A:- BHT, 205, 177,220,206 3A:- THHQ, 123,151,166,124

deterioration of the sensory properties of the food products. Auto-oxidation of fats and oils in processed foods can be prevented by the use of oxidation inhibitors or antioxidants. Although the use of antioxidants is well established in history, the development of synthetic antioxidants and their high efficiency even at low concentrations in the inhibition of the oxidation of fat has lead to their widespread use in ready to eat meals. Therefore, many synthetic antioxidants such as butylated hydroxyanisole (BHA), 5-di-*tert*-butyl-4-hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) are used in edible vegetable oil and cosmetics[1,2]. Although they are powerful in protecting product quality in food distribution, excess antioxidants added to food might produce toxicities or mutagenicities, and thus endanger the health of people[3,4]. In the United States and codex alimentarius commission[5], 2- and 3-*tert*-butyl-4- hydroxyanisole (BHA), 3, 5-di-*tert*-butyl-4-hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) may be used individually or in combination up to a maximum limit of 200 mg kg^{-1}. In Europe, the use of antioxidants is regulated by Directive No. 95/2/EC[6]. In most countries, the content of phenolic antioxidants in processed food is strictly limited. Many manufacturers prefer to use synthetic antioxidants because they are stable, cheap and easily available. However, these synthetic antioxidants have potential risks[7] raising safety concerns in a growing number of consumers[8-11]. Regarding this aspect of safety and hygiene for foods, it is predictable that there will be stricter limits on the use of synthetic antioxidants in the future. The methods mainly reported for the determination of antioxidants and preservatives in Foods, cosmetics and pharmaceuticals were based on high performance liquid chromatography (HPLC)[12-14] gas chromatography (GC)[15-18], ion chromatography (IC)[19] capillary electrophoresis (CE)[20-24] high performance liquid chromatography electrospray mass spectrometry (HPLC–MS)[25] and gas chromatography– mass spectrometry (GC–MS)[2]. Most of study in foods based on edible vegetable oil but very few studies have been reported for analysis of SPA in ready to eat food. However, to the best of our knowledge, so

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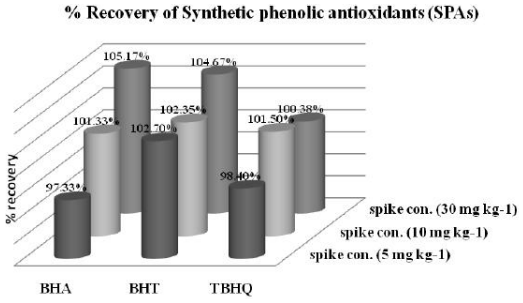

Figure 2 : % Recovery of Synthetic phenolic antioxidants (SPAs)

far no person has reported the method for determination of antioxidants in vegetarian ready to eat meals. So we are including such kind of sample for study. The purpose of the present study was to develop a simple, reliable and sensitive method for the simultaneous determination and confirmation of the three compounds in vegetarian ready to eat meals product by GCMS with simple sample preparation. Main focus was to simplify the analytical process as much as possible during extraction without sacrificing high recoveries for analytes. This proposed method optimises all parameters for determination at the range of its permitted limit in different food product.

EXPERIMENTAL

Chemicals

Reference compounds butylated hydroxyanisole (BHA, CAS No.-25013-16-5), tertiary butylhydroquinone (TBHQ, CAS No.-1948-33-0), butylated hydroxytoluene (BHT, CAS No.-128-37-0) was purchased from Fluka (Buchs, Switzerland) and Aldrich (Sigma-Aldrich, Steinheim, Germany). All reference compounds have a purity of P 98.0%. HPLC grades Methanol and ethyl acetate were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA).

Instrument

GC/MS

A Varian 3200 GC gas chromatograph equipped with a programmed split/split less injector, 88000 auto injector, and a Varian 2000 ion Trap mass selective detector (Varian, Inc. CA, USA) were used to perform all GC analyses. A VF-5MS (30 m × 0.25 mm id, 0.25 µm thickness) column was used. Helium was used as the carrier gas at a flow rate of 1 ml/min. The injector temperature was 80°C for 1 min. Then 280C at 200°C/ min. maintain for 35 min., samples were injected in the split less mode, and the injection volume was 5 µl. SPA are low temperature eluting, all compounds eluted up to 180°C so oven temperature was programmed as follows: 80°C for 3 min, then 150°C at 15°C/min, maintained for 5 min., 180°C at 5°C /min., 280 at 25°C / min., maintained for 12.33 min.

The mass spectra were obtained using the mass selective detector under electron impact ionization at a voltage of 70 eV, mass operating condition are as follow Trap Temperature 200°C , Manifold temperature 80°C and Transfer line temperature 230°C and data acquisition was at a scan rate of 0.95/s over an m/z range of 50-500. Chemist 6.42 software (Varian, Inc. CA, USA) was used to process peak areas. The confirmation of SPA was performed by comparing the observed mass spectra with those recorded in standard spectrometry libraries and by identical retention time of a standard injected under the same conditions.

Standard preparation

0.1 g (accurately weighed to 0.0001 g) of all reference compounds were individually weighed into a 100 mL amber volumetric flask and dissolved with methanol. Standard mixtures of all analytes at different concentration levels were prepared in ethyl acetate to establish the linearity range and the calibration curves.

Sample preparation

Representative food items were chosen in an attempt to cover the analytical complexity of food matrixes. In these groups different food items were tested in order to evaluate possible interferences. Total Nineteen test samples of ready to eat food (Steamed & Fried Rice, Rajma masala (kidney beans), Aloo mutter (potato & green peas), Dal makhani (pulses prepared in butter), Chana dal (dish made using hulled, split hickpeas), Palak Paneer (cheese-based dishes)) item were purchased from local markets in Delhi, India.

Taken 1.00 gram homogenised sample with 5 ml water, 1 gram NaCl and 5 gram sodium Sulfate, add 10 ml ethyl acetate homogenised it at 5000 rpm for 2

min and centrifuge at 12000 rpm at 5°C for 10 min. After centrifuge upper layer of ethyl acetate are taken for GCMS analysis in vial.

RESULTS AND DISCUSSION

Phenolic antioxidant especially TBHQ is readily decomposed by oxygen in air. It is therefore necessary that their concentration be determined immediately after preparation of the sample solution. Whereas BHA, BHT in standards are stable.

Extraction method

Usually the simplest way to improve efficiency of analytical method is to reduce sample size to minimum amount that will provide statistically reliable results, while taking into account the degree of sample homogeneity and scale the method accordingly.

AOAC methods 968.17 and 983.15[26] and other method[27,28] recommended sample preparation and clean up procedures are based on Cereals and vegetable oils and they are show high noise, low sensitivity, loss of analyte , use of large volume of organic solvent and co-eluting compound on GCMS and time taken. For avoided such kind of problem We have optimise Several parameters for achieving detection range up to permitted limited take. 0.1 gram equivalent weight of sample for analysis and eliminate solvent evaporation step from sample preparation.

Optimization of extraction

Several parameters (sample amount, homogenization method, extraction solvents, extraction volume, and number of steps) were studied in order to optimize the extraction procedure. Different amounts of sample (1, 2, and 5 g) were tested, and the best results were obtained with 1.0 g sample. Homogenization is an important step because it can significantly affect the recovery of the method.

Ethyl acetate was used as extraction solvent. The solvent volume and number of extractions were evaluated. Best results were achieved when extraction was performed with 10 mL ethyl acetate and homogenised for 2 min.

SPE cleanup

During this study we are also seen dispersive solid phase extraction as another alternative for cleanup. C18, PSA were trying for that but they were show loss of analyte during recovery study in different level.

TABLE 1 : Recovery & calibration curve parameters

Parameters			
Compound	TBHQ	BHA	BHT
Correlation coefficient (R^2)	0.998	0.999	0.999
Retention Time (min.)	13.77	11.88	12.11
Diagnostic ions (m/z)	123#,151, 166,124	165#,137, 180,166	205#,177 ,220,206
Linearity (working range), mg l^{-1}	0.1-5.0	0.1-5.0	0.1-5.0
Detection limit, mg kg^{-1}	0.1	0.1	0.05
Repeatability (% RSD at 5 mg l^{-1} con.)	6.73	7.84	9.67
Maximum permitted level (mg kg^{-1})*	200	200	200
Recovery in Ready to eat food product (mean Recovery % (n=6) ±SD)			
Fortification level (mg kg^{-1})	TBHQ	BHA	BHT
5	98.4±6.64	97.3±7.63	102.7± 9.93
10	101.5±7.20	101.3±7.97	102.4± 9.99
30	100.4±6.86	105.2±11.29	104.7± 7.66

***As per codex alimentarius commission. general standard for food additives CODEX STAN 192-1995 (Rev. 6-2005),#Quantification ion**

Optimization of chromatographic methods

With the GC method, several preliminary studies were performed in order to obtain a fully resolved peak for SPA. The GC method was optimized, testing the effect of the injection mode (split or split less) and the temperature gradient program on the SPA peak resolution. Best results were achieved using split less injection. During study we found carrier over was show when sample were highly contaminant (=400 ppm) and carrier over amount is less than 0.1 %. The GC column should be cleaned properly (using an isocratic oven program of 280°C for 12 min) between successive injections to avoid carryover from strongly contaminated solutions. A chromatogram in the full scan mode is shown in figure 1.

Linearity

To carry out this study, matrix match standards with six levels of concentration within the range of 0.1-5 mg kg^{-1} were prepared. Analysis was performed in triplicate. Quantitation was carried out using the peak area from the chromatogram of the molecule. The linearity range, correlation coefficient were listed in TABLE 1.

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As observed, the linearity of the analytical response within the studied range of 2 orders of magnitude is excellent, with correlation coefficients higher than 0.998 in all cases.

Precision

Assay precision of the method was evaluated by analysis of six replicates of standard solution for three concentrations. The precision was between 6.82% to 10.74%. The result shows good reproducibility and precision of method.

Recovery

The recovery of the method was studied at the concentration levels of 5, 10 & 30 mg kg^{-1} where a known concentration of the analytes was added to a determined amount of placebo and it was calculated by the concentration of the analytes recovered in relation to that added. The results obtained for the accuracy study (recovery method) from samples (n = 6 for each concentration level) are presented in TABLE 1 and figure 2. As shown in TABLE 1, it can be concluded that the recovery study of the antioxidants and preservatives in the ready to eat food product was correct, therefore, the proposed analytical method was sufficiently accurate.

Limits of detection

The limits of detection (LODs) were estimated with concentration levels giving a signal-to-noise ratio of about 3.The results are shown in TABLE 1. As observed, the method limits of detection obtained are lower than the maximum residue level established for these antioxidants and preservatives.

Selectivity using GCMS

Selectivity is the ability to separate or isolate the response of the target compounds from matrix interferences. Retention Time of analyte shift between ±0.07 shows during repeatable analysis. High selectivity for the target compounds can be achieved during analysis.

Sample determination

To evaluate the effectiveness of the proposed method, it was applied to the analysis of a total of nineteen test samples of ready to eat food item. Fortunately, in most cases, most antioxidants and preservatives were not found. Only BHA in 6 samples in range of 8-26 ppm and TBHQ in one sample 2ppm were detected but all are also below MRL.

CONCLUSION

A new analytical method has been developed and applied in routine for screening quantitation and confirmation of antioxidants and preservatives in ready to eat food items in the range of concentrations between 0.1 and 400 mg/kg, and had show good linearity and low detection limits. This method show significant role especially routine analysis of ready to eat food product because it's simple, cheap and rapid sample preparation method.

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