Butylated hydroxytoluene (BHT)

Application	Determination in urine
Analytical principle	Capillary gas chromatography/mass spectrometric detection (GC/MS)
Completed in	December 2005

Summary

The analytical procedure described here serves to quantify butylated hydroxytoluene (BHT) in the urine of persons exposed to BHT at the workplace.
After the urine sample has been hydrolysed with hydrochloric acid for one hour at 100 °C, its pH is adjusted to the alkaline range with sodium hydroxide, and then it is extracted with toluene. The analysis of BHT is performed by gas chromatography with a mass spectrometric detector. External calibration is carried out using standard solutions that are prepared in urine. The calibration solutions are processed and analysed in the same manner as the samples.

Butylated hydroxytoluene (BHT)

Intra-assay repeatability:	Standard deviation (rel.)	s_w = 6.7% or 1.5%
	Confidence interval	u = 17.2% or 3.9%
	at a concentration of 10 or 100 µg BHT per litre urine and where n = 6 determinations in each case	
Inter-day repeatability:	Standard deviation (rel.)	s_w = 8.5% or 5.7%
	Confidence interval	u = 21.8% or 14.6%
	at a concentration of 10 or 100 µg BHT per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 110% or 97%
	at a spiked concentration of 50 µg or 200 µg per litre urine determined in n = 10 individual urine samples	
Quantification limit:	3 µg BHT per litre urine	

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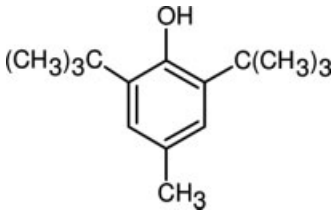

Due to its radical capturing properties, butylated hydroxytoluene (BHT; synonyms: 2,6-di-*tert*-butyl-4-methylphenol, 2,6-di-*tert*-butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxytoluene) is used as an antioxidant in various areas (as a stabiliser in the manufacture of plastics, an anti-ageing agent for plastic and rubber articles, an oxidation protection agent in adhesives, lubricating oils, cosmetic preparations and food). BHT is therefore ubiquitously present in the environment. The global production of BHT was approx. 62 000 tonnes in the year 2000 [1].

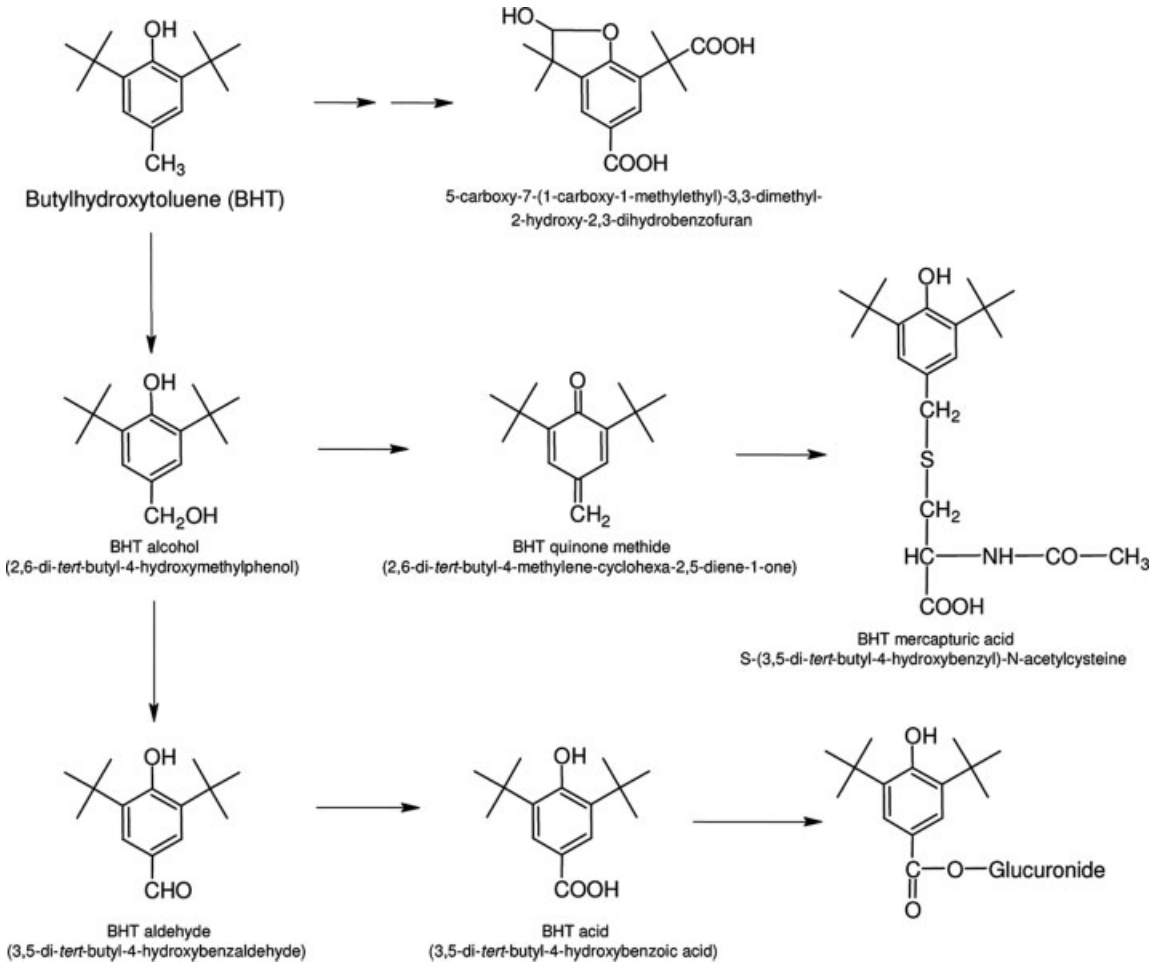

Fig. 1. Simplified diagram of the metabolism of BHT according to [2, 3].

A comprehensive review of the toxicological aspects of BHT can be found in the justification for the MAK value [2, 3]. The Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area has assigned BHT to the Category 4 group of carcinogens. The MAK (maximum permissible concentration at the workplace) value for BHT is 20 mg/m^3 [4]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has derived a value of 0 to 0.3 mg per kg body weight as the acceptable daily intake (ADI) for BHT, which is registered as a food additive with an antioxidant effect [5].

The main route of intake at the workplace is via inhalation. Less than 1% of the dose is absorbed through the skin. Approx. 75% of a single oral dose of 40 mg ^{14}C-BHT administered to humans was recovered after 11 days in the urine, with half of it being recovered within the first 24 hours. Several metabolic pathways and numerous metabolites are known for BHT [2, 3] (see Fig. 1). One of the breakdown routes involves stepwise oxidation of the 4-methyl group to form BHT alcohol, BHT aldehyde and BHT acid and its glucuronides. In human metabolism only about 3% of the dose is degraded to the acid. 5-Carboxy-7-(1-carboxy-1-methylethyl)-3,3-dimethyl-2-hydroxy-2,3-dihydrobenzofuran yielded by ring formation and oxidation is under discussion as the main metabolite. Furthermore, it has already been confirmed that BHT can also be oxidised by cytochrome P450 via a BHT phenoxy radical to a BHT quinone methide (2,6-di-*tert*-butyl-4-methylene-cyclohexa-2,5-diene-1-one). The quinone methide is further metabolised to mercapturic acid through conjugation.

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Contents

1 General principles

The urine samples are subjected to hydrolysis with hydrochloric acid for one hour at 100 °C. After the pH has been adjusted to the alkaline range using sodium hydroxide, BHT is extracted with toluene. Then BHT is analysed by means of gas chromatogra-

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phy with a mass spectrometric detector. External calibration is carried out using standard solutions that are prepared in urine. The calibration solutions are processed and analysed in the same manner as the samples.

2 Equipment, chemicals and solutions

2.1 Equipment

Gas chromatograph with split/splitless injector, mass selective detector, autosampler and data processing system

Capillary gas chromatographic column:
Stationary phase: (5% phenyl)methylpolysiloxane; length: 25 m; inner diameter: 0.2 mm, film thickness: 0.33 µm (e.g. DB-5, Agilent J&W No. 128-5022)

Double gooseneck liner (e.g. Restek)

Analytical balance

500 mL Glass beaker

10 and 500 mL Volumetric flasks

Polypropylene vessels for collecting urine (e.g Kautex No. 303-783859)

Variable piston pipettes for volumes between 10 µL and 1000 µL (e.g. Brand)

Hand dispenser for volumes between 250 µL and 500 µL (e.g. Brand)

10 mL Test tubes with Teflon-coated screw caps, new (cf. Section 9.4) (e.g. Schütt or O. Kohl – Ritterhude)

Alternatively: Sealable test tubes made of Teflon (e.g. Nalgene)

Dry thermostat or drying cupboard (e.g. Heraeus)

Laboratory shaker (e.g. IKA Vibrax VXR)

Laboratory centrifuge (e.g. Heraeus)

Autosampler vials, N 08-02, 300 µL (e.g. Macherey-Nagel)

Crimp caps R-8 PTFE, Teflon septa (e.g. CS-Chromatographie Service GmbH)

Teflon adapter for N 08-02 autosampler vials (e.g. Chromacol)

2.2 Chemicals

Sodium hydroxide, p. a. (e.g. Fluka No. 30620)

Ultrapure water (e.g. Milli-Q water)

2,6-Di-*tert*-butyl-p-cresol (BHT) (e.g. Fluka Art. No. 34750)

Acetonitrile SupraSolv® (e.g. Merck No. 100017)

37% Hydrochloric acid, p. a. (e.g. Merck No. 100317)

Toluene for trace analysis (e.g. Fluka, No. 89676)

Helium 5.0 (e.g. Linde)

2.3 Solutions

Sodium hydroxide solution (7.5 M):
150 g of sodium hydroxide are weighed into a 500 mL glass beaker and dissolved with approx. 300 mL of ultrapure water. Then the solution is transferred to a 500 mL volumetric flask, which is filled to its nominal volume with ultrapure water.

2.4 Calibration standards

Stock solution:
Approximately 10 mg of BHT are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (1 g/L).

Working solution A:
0.1 mL of the stock solution is pipetted into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (10 mg/L).

Working solution B:
1 mL of working solution A are pipetted into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (1 mg/L).

Working solution C:
0.1 mL of working solution A are pipetted into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (0.1 mg/L).

The stock solution is stable for at least 6 months when kept in a refrigerator at approx. 4 °C. The working solutions must be freshly prepared every week.
The calibration standard solutions for the assay of BHT in urine are prepared for each analytical series in pooled urine from persons who have not handled BHT at the workplace. For this purpose 1 mL of urine is placed in a 10 mL test-tube in each case (see Section 9.4) and the relevant volumes of working solutions A, B or C are added as listed in the pipetting scheme in Table 1. The resulting standard solutions in urine are then directly subjected to the same work-up as the samples as described in Section 3.2.

Table 1. Pipetting scheme for the preparation of the calibration standard solutions in urine.

Volume of working solutions			Urine [mL]	Concentration of the calibration standards [µg/L]
A [µL]	B [µL]	C [µL]		
–	–	10	1	1
–	–	20	1	2
–	–	50	1	5
–	–	100	1	10
–	20	–	1	20
–	50	–	1	50
–	100	–	1	100
20	–	–	1	200
50	–	–	1	500
100	–	–	1	1000

3 Specimen collection and sample preparation

3.1 Specimen collection

The urine is collected in polypropylene vessels, and then analysed immediately if possible. If the analysis cannot be performed immediately, then the urine can be stored deep-frozen at a temperature of about –20 °C for at least 6 months.

3.2 Sample preparation

The urine sample is homogenised by manually shaking the vessel. Then a pipette is used to transfer 1 mL of the urine sample into a new (see Section 9.4) 10 mL test tube with a Teflon-coated screw cap or alternatively into a sealable test tube made from Teflon. After 250 µL of concentrated hydrochloric acid has been added, the sample is then hydrolysed for one hour at a temperature of 100 °C in a dry thermostat or drying cupboard. After the sample has cooled down, 0.5 mL of sodium hydroxide solution (7.5 M) is added (pH > 11). When the sample has cooled down to room temperature, 0.3 mL of toluene is added. The sample is extracted for 10 minutes on a laboratory shaker. Then the samples are centrifuged at 2200 g for 5 minutes. 200 µL of the upper organic phase are transferred to an autosampler vial, which is then sealed using a crimp cap with a Teflon septum.

4 Operational parameters

The analytical measurements are performed on a gas chromatograph coupled with a mass spectrometer.

4.1 Operational parameters for gas chromatography

Capillary column:	Material:	Fused silica
	Stationary phase:	DB-5
	Length:	25 m
	Inner diameter:	0.2 mm
	Film thickness:	0.33 µm
Temperatures:	Column:	Initial temperature 80 °C, 1 min isothermal, then increase at a rate of 12 °C/min to 150 °C, then at a rate of 30 °C/min to 320 °C, then 5 min at the final temperature
	Injector:	300 °C
	Transfer line:	250 °C
Injector liner:	Double gooseneck liner	
Carrier gas:	Helium 5.0, 80 kPa pre-pressure (isobar)	
Split:	Split off for 1 min, then 40 mL/min	
Septum purging:	3 mL/min	
Sample volume:	1 µL	

4.2 Operational parameters for mass spectrometry

Ionisation type:	Electron impact ionisation (EI)
Ionisation energy:	70 eV
Source temperature:	180 °C
Dwell time:	80 ms
Electron multiplier:	2200 V

All other parameters must be optimised in accordance with the manufacturer's instructions.

5 Analytical determination

In each case 1 µL of the toluene extract of the urine sample processed as described in Section 3.2 is injected splitless into the GC/MS system for gas chromatographic analysis. At least one urine control sample (see Section 8) and one reagent blank are included in each analytical series. Ultrapure water serves as a blank instead of urine, and it is subjected to the processing described above. Samples with a content of

Table 2. Retention time and recorded mass of BHT.

Analyte	Retention time [min]	Quantifier [m/z]	Qualifier [m/z]
BHT	9.8	205	220

greater than 1000 µg/L are appropriately diluted with ultrapure water, processed anew and measured. The temporal profiles of the ion traces shown in Table 2 are recorded in the SIM mode in the mass-selective detector.
The retention time for BHT shown in Table 2 serves only as a guide. Users of the method must satisfy themselves of the separation power of the capillary column used and the resulting retention behaviour of BHT. Figure 2 shows an example of a chromatogram of a urine sample spiked with 10 µg BHT per litre. Figure 3 shows the EI mass spectrum of BHT.

6 Calibration

The calibration standard solutions prepared in urine as described in Section 2.4 are processed in the same manner as the urine samples (described in Section 3.2) and analysed by gas chromatography/mass spectrometry as stipulated in Sections 4 and 5. The linear calibration graph is obtained for external calibration by plotting the peak areas of BHT as a function of the spiked concentrations. A linear calibration graph comprising at least six calibration points should be plotted for each analytical series. The linearity of the analytical method is given in the concentration range of 1 to 1000 µg/L. Figure 4 shows a calibration curve in this range for BHT prepared in urine.

7 Calculation of the analytical result

The BHT concentration in a sample is calculated by means of external calibration on the basis of the linear calibration graph obtained as described in Section 6. The relevant concentration in µg/L urine is determined from the peak area of the analyte in the analytical sample via the calibration function. Any blank value measured must be taken into account.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* [German Medical Association] [6] and in the special preliminary remarks to this series.

At least one control sample of urine is analysed with each analytical series in order to check the precision. As quality control material for BHT in urine is not commer-

cially available, it must be prepared in the laboratory. For this purpose urine from persons who have not handled BHT at the workplace is spiked with a defined quantity of BHT in the relevant concentration range (e.g. 10 µg/L). The control urine is divided into aliquots, and can then be stored in the deep-freezer at a temperature of about -20 °C for at least six months. The theoretical value and the tolerance ranges of this quality control material are ascertained in a pre-analytical period (one analysis of the control material on each of 15 different days) [6–8].

9 Evaluation of the method

9.1 Precision

To determine the intra-assay repeatability, urine from persons who were not occupationally exposed to BHT was spiked with a concentration of 10 and 100 µg BHT per litre respectively. Then the spiked urine samples were each analysed six times. Table 3 shows the values for intra-assay repeatability that were obtained.

Table 3. Intra-assay repeatability for the assay of BHT in urine (n=6).

Concentration of BHT [µg/L]	Standard deviation (rel) s_w [%]	Confidence interval u [%]
10	6.7	17.2
100	1.5	3.9

The inter-day repeatability was determined by analysing the same urine samples spiked with 10 and 100 µg BHT per litre respectively on six working days. The precision results are shown in Table 4.

Table 4. Inter-day repeatability for the assay of BHT in urine (n=6).

Concentration of BHT [µg/L]	Standard deviation (rel) s_w [%]	Confidence interval u [%]
10	8.5	21.8
100	5.7	14.6

9.2 Accuracy

The relative recovery rates shown in Table 5 for the two spiked concentrations of 10 and 100 µg BHT per litre urine were calculated from the analyses performed to determine the intra-assay repeatability and the inter-day repeatability.

Table 5. Relative recovery of BHT in urine (n = 6).

Spiked concentration [µg/L]	Intra-assay repeatability		Inter-day repeatability	
	Relative recovery *r* [%]	Range [%]	Relative recovery *r* [%]	Range [%]
10	99.5	91 – 107	105	95 – 117
100	100	99 – 102	92.0	85 – 97

In addition, one of the examiners carried out recovery experiments on 10 individual samples of urine that had been spiked with concentrations of 50 and 200 µg/L respectively. The relative recovery rates thus obtained are presented in Table 6.

Table 6. Relative recovery rates for BHT in spiked individual samples of urine (n = 10).

Spiked concentration [µg/L]	Relative recovery *r* [%]	Range [%]	Standard deviation (rel) s_w [%]
50	110	105–119	4.1
200	97	83–107	7.4

9.3 Detection limit and quantification limit

The detection limit was estimated as three times the signal/background noise ratio in the temporal vicinity of the analyte signal. The detection limit for BHT was approx. 1 µg/L under the conditions described above. The quantification limit was about 3 µg per litre urine and was estimated as nine times the signal/background noise ratio.

9.4 Sources of error

During development and examination of the method outliers were repeatedly observed when glass test-tubes that had been in use in the laboratory for a longer period of time were employed in the test series to determine the precision. The authors suspected that this was due to adsorption effects of BHT on the glass walls that had been attacked by washing cycles. In contrast, when new, unused glass test-tubes or test-tubes made of Teflon were employed, no outliers were observed and readily re-

producible results were obtained. Another possibility is to use glass test-tubes exclusively for the analysis of BHT and to wash them by hand.
Furthermore, high blank values were observed in some cases, especially by one examiner (up to 20 µg/L). The cause of this was identified as the butyl rubber septa of the GC vial sealing caps. In subsequent tests the authors were able to show that increasing concentrations of BHT were to be observed when injections were repeated from the same GC vial that was sealed with such a septum. Depending on the manufacturing batch and the type of solvent, low to very high BHT amounts may be liberated from these septa. Therefore only sealing caps with Teflon septa should be used for the GC vials.
After elimination of the identified sources of error, the remaining reagent blank value was in the range of the detection limit at approx. 1 µg/L.

10 Discussion of the method

Assay of BHT in the urine of occupationally exposed persons is possible with the analytical method presented here. The analysis can be rapidly carried out with the widely available GC/MS analytical technique. Due to the simple sample processing it is possible to work up at least 48 samples per working day, even for inexperienced laboratory staff.
Chromatographic interference caused by the urine matrix was not observed, despite the omission of laborious clean-up steps (see Figure 2). In view of the use of external calibration, the precision and the recovery of the method are considered good. Investigations of spiked individual urine samples showed no indication of a notable matrix dependency.
During optimisation of the method significant analytical interfering factors, such as adsorption of BHT on the glass or contamination due to the vial septa, were identified and solutions were devised. After this interference had been eliminated, the examiners were able to confirm the reliability criteria of the procedure.
A DB 17 HT capillary column (30 m×0,25 mm×0.15 µm; Agilent J&W) and an HP-5ms capillary column (30 m×0,25 mm×0.25 µm; Agilent J&W) were also successfully used during validation and examination of the method. These columns can be used as alternatives to the specified DB 5 capillary column.

Instruments used:
HP 7673 autosampler, HP 5890 II gas chromatograph, MSD 5970 mass spectrometer, all from Agilent.

11 References

[1] *IPCS INCHEM International Programme on Chemical Safety:* 2,6-Di-*tert*-Butyl-p-Cresol, Screening Information Data Set – SIDs (2005). (http://www.inchem.org/documents/sids/sids/128370.pdf).
[2] *DFG Deutsche Forschungsgemeinschaft:* Toxikologisch-arbeitsmedizinische Begründung von MAK Werten: Butylhydroxytoluol (BHT), 11th issue, VCH, Weinheim (1985).

[3] *DFG Deutsche Forschungsgemeinschaft:* Toxikologisch-arbeitsmedizinische Begründungen von MAK-Werten: Butylhydroxytoluol (BHT) – Nachtrag, 39th issue, VCH, Weinheim (2004).
[4] *DFG Deutsche Forschungsgemeinschaft*: List of MAK und BAT values 2009, Report 45, Wiley-VCH, Weinheim (2009).
[5] *JECFA Joint FAO/WHO Expert Committee on Food Additives:* Butylated Hydroxytoluene (2006). (http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-069.pdf).
[6] *Bundesärztekammer:* Richtlinie der Bundesärztekammer zur Qualitätssicherung quantitativer laboratoriumsmedizinischer Untersuchungen. Dt. Ärztebl. 105, A341–A355 (2008).
[7] *J. Angerer and G. Lehnert:* Anforderungen an arbeitsmedizinisch-toxikologische Analysen – Stand der Technik. Dt. Ärztebl. 37, C1753-C1760 (1997).
[8] *J. Angerer, T. Göen, G. Lehnert:* Mindestanforderungen an die Qualität von umweltmedizinisch-toxikologischen Analysen. Umweltmed. Forsch. Prax. 3, 307–312 (1998).

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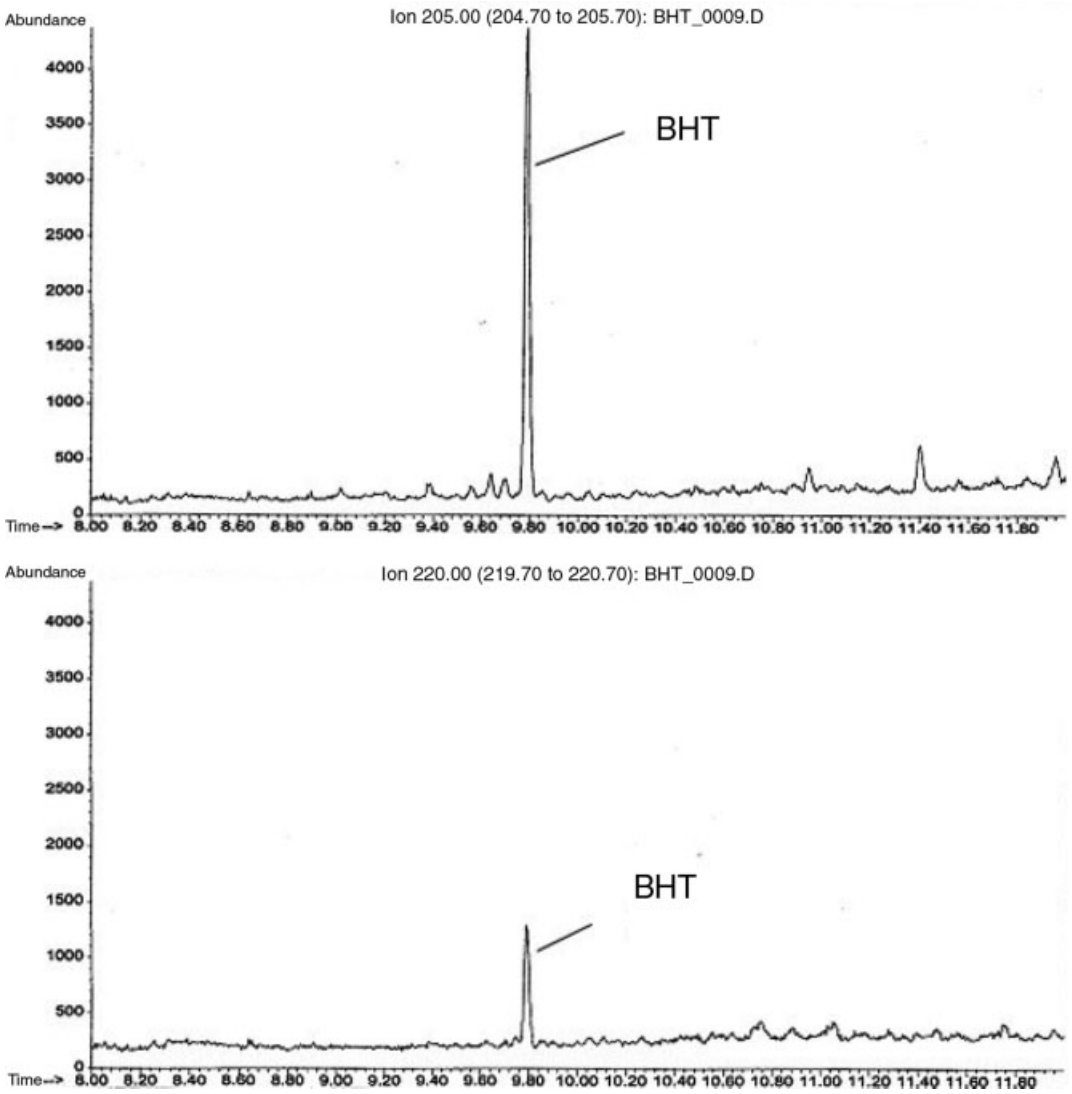

Fig. 2. GC/MS-SIM chromatogram (EI mode) of a urine sample spiked with 10 µg BHT per litre (top: quantifier profile; bottom: qualifier profile).

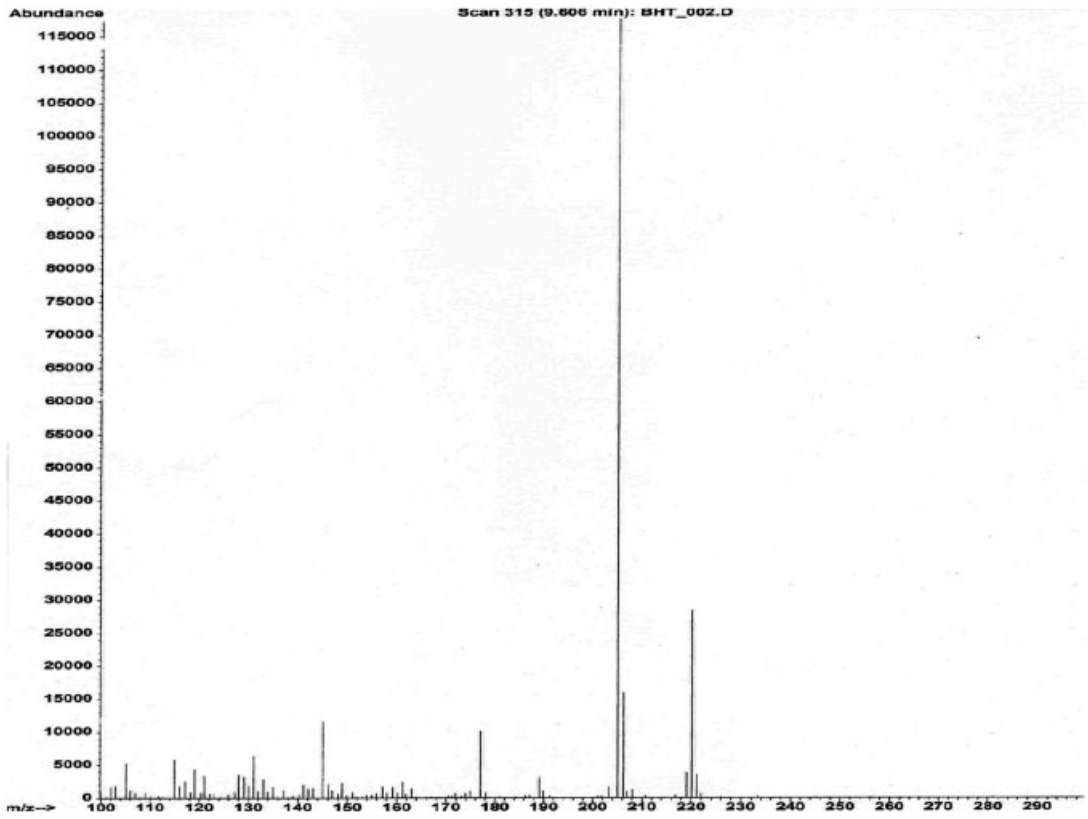

Fig. 3. EI mass spectrum of BHT.

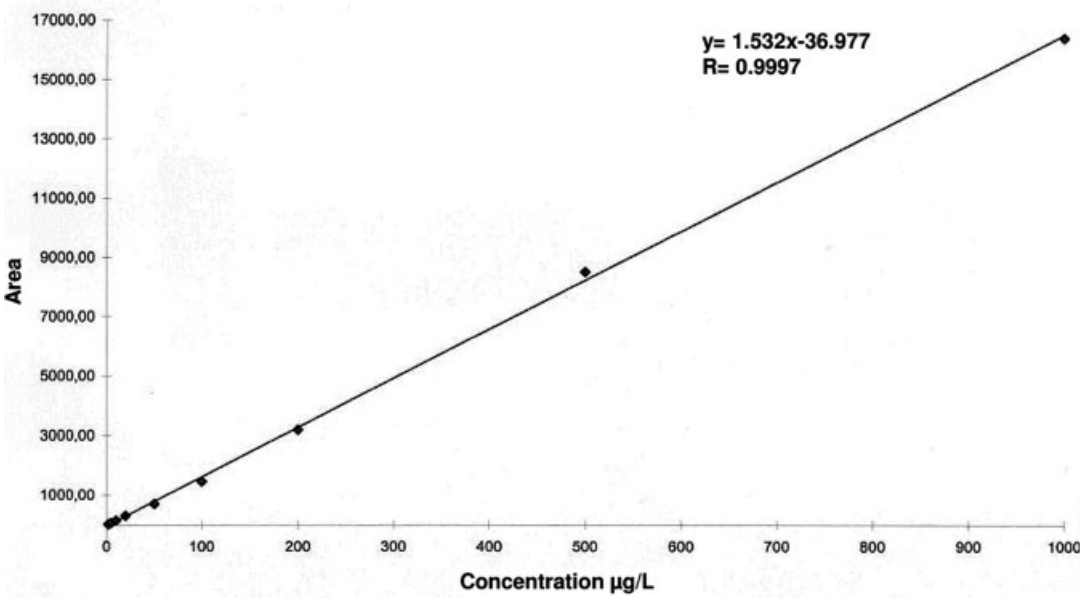

Fig. 4. Calibration curve of BHT in urine.