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# Counterfeit homeopathic medicinal products: Syrups A simple and rapid LC–ESI-MS method to detect preservatives not declared in label

Alessia Panusa*, Luigi Gagliardi

*Dipartimento del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

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ABSTRACT

A rapid and simple LC–ESI-MS method for the simultaneous detection and quantitation of six preservatives in homeopathic syrups has been developed. Counterfeit homeopathic syrups are suspected to contain preservatives that are not declared in label. For this reason a method to ascertain the absence of sorbic and benzoic acids, methyl-, ethyl-, propyl- and butyl-parabens, as the most frequently utilised preservatives, has been developed. Analytes were eluted with a linear gradient of acetonitrile–5 mM ammonium acetate in 12 min using 2,4-dichlorobenzylalchol as Internal Standard. The HPLC separation was performed on an Eclipse XDB-C18 (2.1 mm × 50 mm-5 μm) column and the ESI-MS detection was performed in negative ion mode. Linearity of the method was studied in the range of 2 pg to 10 ng injected and correlation coefficients $r^2 \geq 0.9992$ were obtained. LOD ranged from 0.04 to 0.4 ng $mL^{-1}$.



## 1. Introduction

A preservative is a chemical agent which is included in preparations to prevent deterioration from oxidation (antioxidants) or to kill or inhibit the growth of micro-organisms (antimicrobial preservative) inadvertently introduced during manufacture or use.

Antimicrobial preservatives are used in cosmetics, foods, beverages and non-sterile pharmaceutical products such as oral liquids and creams to prevent microbial spoilage.

Hydroxybenzoates (parabens) are alkyl esters of *p*-hydroxybenzoic acid with antibacterial and antifungal properties. While the activity increases with the increase of the alkyl chain length, the aqueous solubility decreases with the increase of the chain. Activity may also be increased by combining two hydroxybenzoates with short alkyl chains. Methyl- and propyl-hydroxybenzoates are used together in some preparations as they act as synergists. Hydroxybenzoates are used as preservatives in pharmaceutical preparations in usual concentration of up to 0.25% [1]. Hypersensitivity reactions have been reported with the use of hydroxybenzoates and recently has been reported that parabens possess oestrogenic activity which increases with the length and the branching of the alkyl ester [2].

The Italian National Institute of Health has initiated a study on the safety of homeopathic medicinal products. The first goal of this project has been to verify that the most frequently utilised FANS had not been fraudulently added in homeopathic preparations [3]. In fact, pharmaceutical counterfeiting is becoming a relevant problem [4–7]. The aim of this study has been to ascertain that preservatives are not fraudulently added in homeopathic syrups. In fact, in homeopathic preparations preservatives are frequently not declared in label. The importance of such a control is due to the fact that homeopathic medicinal products are subjected to medicines manufacturing and labelling regulations [8]. In addition, patients who do not use allopathic medicinal products due to allergic reactions caused by chemicals have been increasingly using such products. For this purpose a simple and rapid method for the detection and quantitation of the most frequently utilised preservatives has been developed. Sorbic acid (SA), benzoic acid (BA), methylparaben (MP) ethylparaben (EP), propylparaben (PP) and butylparaben (BP) have been chosen as the most frequently utilised preservatives. Butylparaben has been included even though it is more frequently used in homeopathic preparations like creams. In literature many articles describe the analysis of these preservatives by HPLC [9–16] although other techniques like TLC [17] and CE [18–20] have been reported. Also the use of LC–MS has been reported [21–26] but as far as we are aware neither articles on the simultaneous analysis of all

* Corresponding author at: Istituto di Chimica Farmaceutica e Tossicologica "Pietro Pratesi", Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milan, Italy. Tel.: +39 02 50319311; fax: +39 02 50319359.
*E-mail addresses:* alessia.panusa@unimi.it, apanusa@iss.it (A. Panusa).

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these preservatives by LC–ESI-MS nor on the analysis of these analytes in homeopathic products have been described. For the analysis of parabens HPLC methods usually require longer gradients than the one that is here depicted owing to the necessity to separate all the analytes before UV detection. In fact, in order to separate completely SA from BA a longer elution and a concentrated buffer should have been utilised [9]. As LC–MS technique allows detection of analytes even if they co-elute [27], a simple and rapid method to separate the six analytes with different lipophilicity in 12 min has been developed. Our study was conducted on homeopathic syrups of different brands available on the market.

## 2. Experimental

### 2.1. Chemicals

All chemical standards were of analytical grade. Methyl 4-hydroxybenzoate (methylparaben, MP, ≥99%), ethyl-4-hydroxybenzoate (ethylparaben, EP, 99%), propyl 4-hydroxybenzoate (propylparaben, PP, 99%), butyl 4-hydroxybenzoate (butylparaben, BP, 99%) and sorbic acid (SA, 99%) were all purchased from Sigma–Fluka–Aldrich (Milan, Italy); benzoic acid (BA, 99.5%) from Carlo Erba (Milan, Italy); 2,4-dichlorobenzylalchol (IS, 99.9%) from Acros (Geel, Belgium). Ammonium acetate (98%) was supplied by MP Biomedicals (Strasbourg, France). HPLC-acetonitrile was supplied by Merck (Darmstadt, Germany). Acetic acid and HiPerSolv water for HPLC were purchased by BDH (Poole, England). Homeopathic syrups analysed were of different brands.

### 2.2. Instrumentation

Analyses were performed using an Agilent Technologies (Palo Alto, CA, USA) LC–ESI-MS system. The HPLC 1100 system was equipped with a binary pump, a vacuum degasser, an autosampler and a diode-array UV–vis detector. The autosampler and column thermostat controls were set at 4 and 25 °C, respectively. The mass spectrometer was equipped with an electrospray (ESI) source connected to a single quadrupole SL model. The Agilent ChemStation software Rev. B.01.03 (204) was used for the data processing and control of the LC–ESI-MS system. Chromatographic separations were performed by reverse phase elution with an Agilent Eclipse XDB-C18 LC/MS (2.1 mm × 50 mm-5 μm) column.

HPLC vials and PTFE screw caps were purchased from Agilent Technologies. Samples were filtered by Acrodisc syringe filters, pore size 0.2 μm.

### 2.3. Standard solutions and sample preparation

Standard stock solutions were prepared at 1 mg mL$^{-1}$ in acetonitrile–water (25:75, v/v) for all analytes with the exception of IS prepared at 2 mg mL$^{-1}$.

All solutions were prepared and dilutions were performed with the same acetonitrile–water (25:75, v/v) percentage. Multicomponent stock solutions were prepared at 1 μg mL$^{-1}$ by dilution of stock solutions. Multicomponent working solutions were prepared in the 0.4 ng mL$^{-1}$ to 2 μg mL$^{-1}$ range by dilution of multicomponent stock solutions or standard stock solutions.

Syrup samples were preliminarily diluted 1:100. Then 2 mL were added with 5 mL of IS, made up to 25 mL and then analysed.

For recovery experiments homemade syrups were prepared with 50 g solubilised with 25 g of water. Before analysis they were diluted 1:100, added with IS as previously reported and spiked with multicomponent solutions. Spiking was performed at concentration of 40 and 400 ng mL$^{-1}$. All samples were prepared in triplicate and filtered before injection.

**Table 1**
Name, formula, relative molecular mass ($M_r$), retention time ($t_r$), mass charge ($m/z$) and fragmentor for each analyte

| Analyte | Formula | $M_r$[a] | $t_r$ | $m/z$ | Fragmentor voltage (V) |
|---|---|---|---|---|---|
| SA | $C_6H_8O_2$ | 112.05 | 0.72 | 111 | 90 (–) |
| BA | $C_7H_6O_2$ | 122.03 | 0.75 | 121 | 90 (–) |
| MP | $C_8H_8O_3$ | 152.04 | 3.30 | 151 | 100 (–) |
| EP | $C_9H_{10}O_3$ | 166.06 | 6.50 | 165 | 100 (–) |
| PP | $C_{10}H_{12}O_3$ | 180.07 | 9.45 | 179 | 120 (–) |
| BP | $C_{11}H_{14}O_3$ | 194.09 | 11.78 | 193 | 120 (–) |
| IS | $C_7H_6OCl_2$ | 175.98 | 9.02 | 175 | 110 (–) |

[a] Isotopic molecular mass.

### 2.4. HPLC and ESI-MS conditions

Separation was carried out with a linear gradient elution with acetonitrile (A)–ammonium acetate 5 mM (B) from 20:80 (A:B, v/v) to 65:35 (A:B, v/v) in 15 min. The mobile phase was restored at the initial composition in 3 min and then the column was equilibrated for 7 min. The flow rate was 0.25 mL min$^{-1}$ and the volume injected was 5 μL. The elution times are shown in Table 1.

ESI interface parameters (negative-ion mode) were set as follows: capillary voltage 4000 V; drying gas (nitrogen) flow 12.0 L min$^{-1}$; drying gas temperature 350 °C; nebulizer pressure 50 psig; fragmentor see Table 1.

Preliminary Flow Injection Analyses (FIA) without column were performed to select the mass operating parameters and the ions to be monitored in Single Ion Monitoring (SIM) mode. In FIA analysis the composition of mobile phase was the composition corresponding to the elution times displayed in Table 1. For each analyte FIA experiments were performed in full scan acquisition over (100–1000 $m/z$) range in both positive and negative ionisation mode. For FIA analysis standard solutions at 10 μg mL$^{-1}$ were injected. On the basis of these experiments the $[M-H]^-$ ion was selected for all analytes.

Analyses were performed in SIM mode. Ions were selected taking into account elution times: $t$ = 0–2 min $m/z$ 111, 121; $t$ = 2–5 min $m/z$ 151; $t$ = 5–8 min $m/z$ 165; $t$ = 8–11 min $m/z$ 175, 179; $t$ = 11–15 min $m/z$ 193.

## 3. Results and discussion

### 3.1. Method development and optimisation

Preliminary experiments were performed with Atmospheric Pressure Chemical Ionisation (APCI) and ESI sources in both polarities in order to select the most appropriate interface. With both sources different mobile phases were tested and the ESI source was always the most sensitive.

Firstly, starting experiments were performed with acetonitrile–water (1% acetic acid) and then with acetonitrile–water. The ionisation was greatly improved without acetic acid. Afterwards, acetonitrile–ammonium acetate at different molarities was tested in both polarities. Negative ionisation mode was always the most sensitive. The best results were obtained with the use of ammonium acetate 5 mM. An example of the percent variation of the $[M-H]^-$ peak area for each analyte with mobile phase composition is displayed in Table 2. With the gradient utilised all analytes were

**Table 2**
Variation of $[M-H]^-$ peak area for each analyte with different (B) mobile phase composition (5 mM ammonium acetate = 100%)

| (B) | SA | BA | MP | EP | PP | BP |
|---|---|---|---|---|---|---|
| $H_2O$ | 23 | 28 | 53 | 48 | 47 | 30 |
| 8 mM | 18 | 21 | 40 | 38 | 40 | 23 |

**Table 3**
Linear regression analysis ($y = ax + b$) of mass injected (pg) ($x$) versus ratios of analyte to IS peak area ($y$); correlation coefficients ($r^2$)

| Analyte | $a$ ($\times 10^{-5}$) | $b$ | $r^2$ |
|---|---|---|---|
| SA | 7.19 ± 0.16 | 0.00035 ± 0.00058 | 0.9999 |
| BA | 9.43 ± 0.54 | 0.0196 ± 0.0018 | 0.9992 |
| MP | 88.62 ± 0.26 | 0.0190 ± 0.0088 | 0.9998 |
| EP | 113.1 ± 0.2 | 0.0096 ± 0.0078 | 0.9999 |
| PP | 168.6 ± 0.3 | −0.0049 ± 0.0091 | 0.9999 |
| BP | 210.3 ± 0.4 | 0.0238 ± 0.0146 | 0.9999 |

**Table 4**
Limit of detection (LOD), precision (R.S.D.%) and recovery

| Analyte | LOD (ng mL$^{-1}$)[a] | Precision[b] | | | Recovery | |
|---|---|---|---|---|---|---|
| | | 40 pg[a] | 200 pg[a] | 2000 pg[a] | 200 pg[a] | 2000 pg[a] |
| SA | 0.4 | 4.0 | 3.6 | 0.6 | 81 | 83 |
| BA | 0.4 | 3.5 | 3.3 | 1.2 | 80 | 82 |
| MP | 0.06 | 1.6 | 2.5 | 1.0 | 85 | 93 |
| EP | 0.08 | 1.0 | 3.1 | 0.8 | 85 | 89 |
| PP | 0.06 | 3.0 | 2.8 | 1.2 | 83 | 88 |
| BP | 0.04 | 0.1 | 1.0 | 3.0 | 83 | 89 |

[a] 5 μL injected.
[b] ($n = 6$) replicates.

eluted within 12 min. Retention times are shown in Table 1. When HPLC with UV detection is employed for these analytes, gradients are usually longer [9] due to necessity to separate all components before detection. The use of the ESI-MS detector and a short chromatographic column allowed a more rapid analysis and also the use of smaller volume of solvents. The co-elution of SA and BA did not affect the accuracy of the method. In fact, in preliminary analysis with single component standard solutions no interfering peaks at the same $t_r$ of the other analytes were detected with Extract Ion Chromatogram (EIC). In addition, the same peak areas were obtained for each analyte in single component standard solutions and in multicomponent standard solutions at the same concentrations.

All analytes displayed a $[M-H]^-$ base peak. Detection was performed in SIM mode and the peak areas were measured by the EIC (Fig. 1).

### 3.2. Method validation

Linearity was studied in the wide range of 0.4 ng mL$^{-1}$ to 2 μg mL$^{-1}$ (2, 4, 20, 40, 100, 200, 400, 800, 2000 and 10,000 pg injected) for all analytes with the exception of SA and BA studied in the range of 4 ng mL$^{-1}$ to 2 μg mL$^{-1}$. Experimental data fitting was performed with linear regression analysis. Ratios of peak area of each analyte to IS were reported versus the mass injected (pg) of the analyte. In all cases very good correlation coefficients ($r^2$) in the range of 0.9992–0.9999 were obtained. Equations and correlation coefficients are shown in Table 3.

Precision was calculated on spiked samples and expressed as the relative standard deviation of replicate measurements ($n = 6$) at three analyte mass concentrations 8, 40 and 400 ng mL$^{-1}$ (40, 200 and 2000 pg injected). R.S.D.% values ranged between 0.1 and 4.0. Results are displayed in Table 4.

Recoveries were estimated using homemade syrups spiked with preservatives at two levels, 40 and 400 ng mL$^{-1}$ (200 and 2000 pg injected). Good recoveries ranging between 80% and 92% were obtained for all analytes. Results are in Table 4.

The limits of detection (LOD) are shown in Table 4. LOD were in the range of 0.4 ng mL$^{-1}$ (2 pg injected) for SA and BA to 0.04 ng mL$^{-1}$ (0.2 pg injected) for BP (signal-to-noise 3:1). For SA this value is lower than the LOD reported in literature in LC–ESI-MS [21]. For BA we did not find literature on the analysis of BA by LC–ESI-MS. Also for methyl- and ethyl-parabens the LOD we obtained are lower than the ones reported in literature [22–24].

The method was successfully applied to the analysis of homeopathic syrups available on the market.

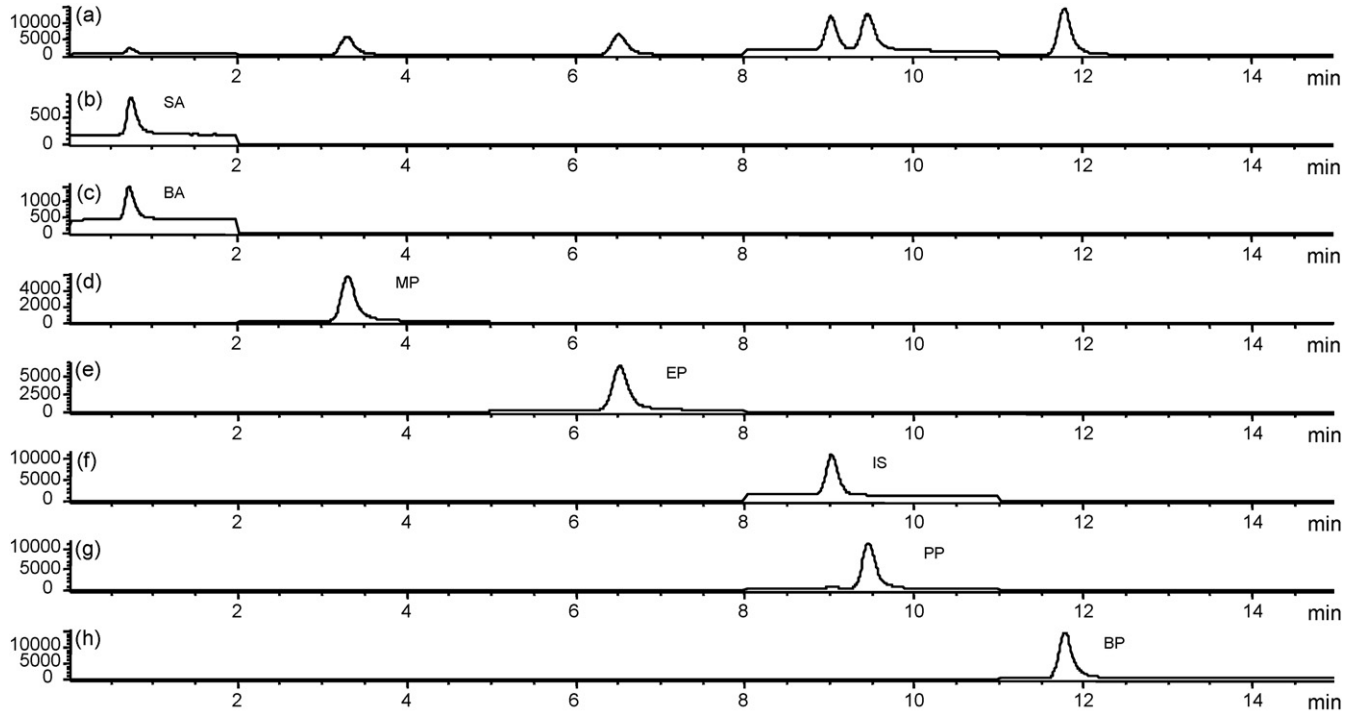


**Fig. 1.** SIM chromatogram of a homemade syrup spiked by SA, BA, MP, EP, PP, BP and IS. (a) TIC. (b) EIC $m/z = 111$ (SA). (c) EIC $m/z = 121$ (BA). (d) EIC $m/z = 151$ (MP). (e) EIC $m/z = 165$ (EP). (f) EIC $m/z = 175$ (IS). (g) EIC $m/z = 179$ (PP). (h) EIC $m/z = 193$ (BP).

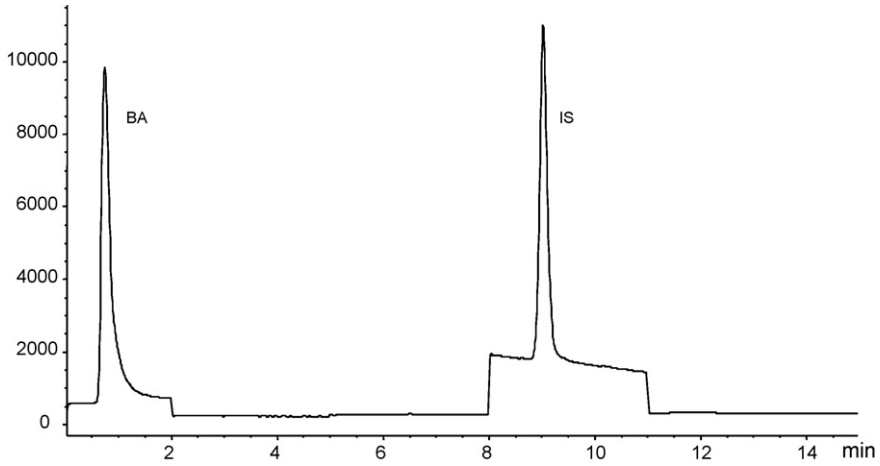


**Fig. 2.** SIM chromatogram of a syrup sample containing BA not declared in label.

Among the syrups we analysed some had preservatives (methyl- and propyl-parabens) declared and other had no preservatives declared on their labels.

The results of our analyses confirmed the presence of declared preservatives for same samples. In other cases preservatives not declared in labels or different from the one declared in label were detected. In Fig. 2 an example of a syrup containing BA not declared in label, is shown. The presence of a peak at $t_r = 0.74$ min revealed the presence of one or both SA and BA. The EIC at $m/z = 111$ and $m/z = 121$ demonstrated that the peak was due to the presence of BA only. In fact, only a peak at $m/z = 121$ was obtained. The result was also confirmed by a full scan acquisition spectrum (100–1000 $m/z$ range) of the syrup sample which matched exactly the preliminary FIA performed on BA standard.

## 4. Conclusion

A LC–ESI-MS method for the detection and quantitation of the most frequently employed preservatives in homeopathic syrups has been developed. SA, BA, MP, EP, PP and BP were eluted in 12 min with a linear gradient of acetonitrile–ammonium acetate 5 mM using 2,4-dichlorobenzylalchol as IS. The detection was performed with an ESI source and a single quadrupole mass spectrometer in negative ionisation mode.

Good linearity in the wide range of 0.4 ng mL$^{-1}$ to 2 μg mL$^{-1}$ (2 pg to 10 ng injected) for analytes was observed with correlation coefficients $r^2 \geq 0.9992$. Repeatability expressed as R.S.D.% of replicates measurements on spiked samples was in the 0.1–4.1 range. LOD from 0.04 to 0.4 ng mL$^{-1}$ (from 0.2 to 2 pg injected) were better than the ones reported in literature.

This simple and rapid LC–ESI-MS method for the detection of the most utilised preservatives has been successfully employed for the screening of homeopathic syrups available on the market.

In same samples preservatives not declared in labels or different from the one declared in label were detected.

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