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Method Development and Validation of the Simultaneous Analysis of Methylisothiazolinone, Methylchloroisothiazolinone, Benzisothiazolinone and Bronopol in Washing-Up Liquid

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Abstract

A method of analysis for the simultaneous determination of methylisothiazolinone (MI), methylchloroisothiazolinone (CMI), benzisothiazolinone (BIT) and Bronopol (BNP) in washing-up liquid was established. The method consisted of a gradient HPLC analysis at three different wavelengths. The four compounds could be analyzed with good precision and accuracy.

Keywords

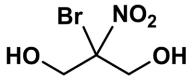
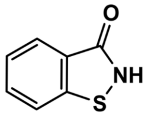
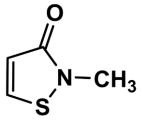
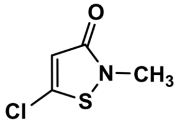
Preservatives, Analytical Validation, Methylisothiazolinone, Methylchloroisothiazolinone, Benzisothiazolinone, Bronopol, HPLC, Washing-Up Liquid

1. Introduction

It is important that a preservative is added to washing-up formulations containing more than about 60% water in order to inhibit bacterial and fungal growth. The most common preservatives for these formulations are 2-bromo-2-nitro-1,3-propanediol, Bronopol (BNP for short); 1,2-benzisothiazol-3(2*H*)-one (BIT); 2-methyl-isothiazol-3-one or methylisothiazolinone (MI) and 5-chloro-2-methyl-isothiazol-3-one or methylchloroisothiazolinone (CMI) (see **Table 1**).

It is imperative that the concentration of these preservatives is closely monitored

Table 1. Names and structures of the four preservatives.

Systematic name	Trivial name	CAS RN	Abbreviation	Structure
2-bromo-2-nitropropane-1,3-diol	Bronopol	52-51-7	BNP	
benzo[*d*]isothiazol-3(2*H*)-one	Benzisothiazolinone	2634-33-5	BIT	
2-methylisothiazol-3(2*H*)-one	Methylisothiazolinone	2682-20-4	MI	
5-chloro-2-methyl-isothiazol-3(2*H*)-one	Methylchloro-isothiazolinone	26172-55-4	CMI	

during the development, production and shelf life of the formulations. It is possible that preservatives react with some of the components of the formulation as evidenced by e.g. a change in the proportion MI/CMI [1]. A decrease in concentration of preservatives over time during shelf life might indicate a problem with plant hygiene [2].

The main problem with these preservatives is, however, that they might induce contact dermatitis. Ample reports can be found, both in the older and the more recent literature. See e.g. for BNP [3]-[8]; CI and CMI [9] [10] [11] [12] [13] and BIT [12] [14] [15] [16]. The use of these preservatives is thus regulated by EU legislation. The maximum allowed concentration of bronopol is 0.1% [17]; for MI and CMI is 0.0015% [17] and for BIT is 0.0005%, *i.e.* 5 ppm [18]. These concentrations apply to ready for use preparations.

For all these reasons mentioned, it is necessary that a fast and simple analysis is available for the chosen preservatives. Apart from a spectrophotometric method for BNP [19] [20], all published methods use HPLC as the preferred method of analysis. Detection can be electro-chemical [21] or UV absorption [22]-[27]. Of course, tandem mass spectrometry can be used as a detection technique as well [28] [29] [30] [31]. To the best of our knowledge, no report has been published in which the four compounds are analyzed simultaneously. So, we set out to find a simple method for the simultaneous analysis of all four preservatives in dish-washer liquid. The method should use a generally available instrument and shouldn't take too much time. Moreover, the method must be suitable for application in other laboratories, so a full method development is required [32] [33].

2. Materials and Methods

2.1. Standards and Reagents

The following standards were used: BIT was from Sigma-Aldrich (Overijse, Bel-

gium) and had a purity of 97%. BNP was purchased from Acros Organics (Geel, Belgium) with a purity of 98%. MI and CMI were from THOR GmbH (Speyer, Germany) as a single solution with 0.377% MI and 1.14% CMI. All standards were used without further purification and the stated purity was used for the calculations.

Acetonitrile, "Far UV"-quality was from Fischer Scientific (Merelbeke, Belgium). Ultra-pure water used for both HPLC and for preparing dilutions was made "in-house" using a Simplicity apparatus from Merck Millipore (Overijse, Belgium).

2.2. Apparatus

The instrument used for the development of the analysis was from Thermo Scientific (Merelbeke, Belgium) and consisted of the following modules: an SCM1000 vacuum degasser; a quaternary gradient P4000 pump with low pressure mixing; an AS1000 auto sampler with a fixed injection volume of 20 µL and a UV6000LP diode array detector with a flow cell volume of 10 µL and an optical path length of 5 cm.

The column used for the separation was a Hypersil GOLD C18 column (Thermo Scientific), with a length of 25 cm and an internal diameter of 0.3 cm, packed with 5 µm particles. (This choice will be discussed in the Results and Discussion section). At regular intervals a blank gradient (*i.e.* injection of pure water) was run before every series of experiments, to ascertain the absence, c.q. identify, peaks originating from the used solvents. However, when using a good quality solvent these blank peaks should be entirely absent or pose no problem for the precision and accuracy of the analysis.

The compounds were separated using a water-acetonitrile (ACN) gradient. The gradient conditions are given in Table 2. The mobile phase flow throughout the entire analysis was 0.5 $mL \cdot min^{-1}$.

2.3. Sample Preparation

All samples were diluted 6 or 25 times (depending on the concentration of the preservatives) using ultra-pure water, followed by filtration over a polyamide membrane with 0.20 µm pores (Chromafil from Macherey-Nagel, Eupen, Belgium) and injection on the HPLC column. In order to obtain a good accuracy, all dilutions were done on a weight basis. Sample and diluting solvent were weighted on an analytical balance (Kern ABS, Balingen, Germany) to the nearest 0.1 mg. The correct diluting factor was then calculated. The analytical balance was properly calibrated using a calibration weight before each series of measurements.

3. Results and Discussion

3.1. Spectra and Detection Wavelength

Figure 1 gives the UV spectra of the four preservatives. The concentrations of the four components in Figure 1 were not the same.

Table 2. Gradient conditions.

Time/(min)	% ACN
0	5
10	50
11	100
13	100
14	5
22	5

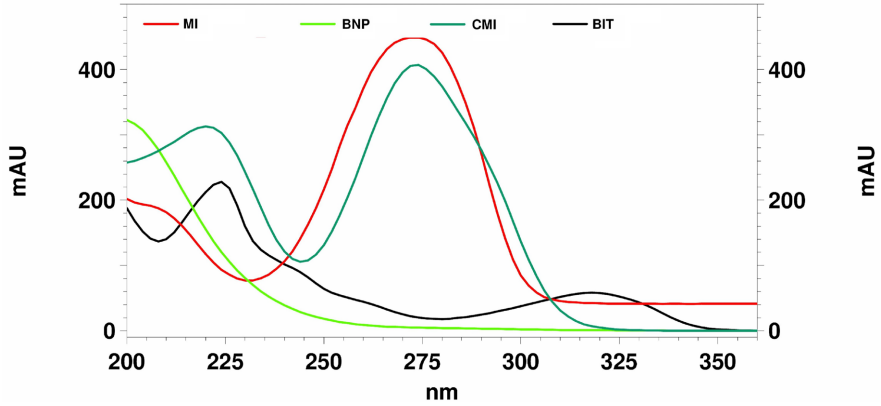


Figure 1. UV spectra of the four preservatives.

Judging from the spectra in Figure 1, it is not possible to detect all four components using a single wavelength without severely compromising the detectability at low concentrations. Therefore, it was decided to use three different wavelengths for the detection of the four components, as our instrument allowed the simultaneous use of up to three different wavelengths for detection. Based on the spectra, the optimum detection wavelengths, together with the retention times, are given in Table 3.

3.2. Choice of the Analytical Column

In a washing-up liquid, the preservatives are dissolved in a matrix that contains up to 30% detergents. These detergents might interfere with the analysis. A preconcentration step was considered, in order to isolate the analytes from the matrix. However, the main matrix components, *i.e.* detergents, do not give univocal specific interactions. They have—by definition—a non-polar and a polar (often charged) part within the molecule. A precolumn sample preparation, such as solid phase extraction (SPE), depends on a single type of intermolecular interaction between a molecule in the sample and the stationary phase. Examples are non-polar interactions or ion exchange equilibria. Such single interactions, however, are impossible with detergent molecules. So, it is impossible to clean up the sample with SPE from possible interfering matrix components, if these components are detergents.

Table 3. Detection wavelength λ_{det} and retention times t_R.

Compound	λ_{det}/(nm)	t_R/(min)
MI	270	4.1
BNP	200	6.8
CMI	270	8.7
BIT	224	10.3

As a consequence, the analytical column will have to be chosen in such a way that there are no matrix interferences at the retention times of the analytes and the wavelengths used.

The columns that were tested during this research are summarized in **Table 4**. As evidenced by the comments, only one column from the several manufacturers and column chemistries that were tested was suitable: the Hypersil Gold C18. So, this was the column of our choice.

3.3. Sample Chromatograms

In **Figure 2** sample chromatograms of the four preservatives at three different detection wavelengths are given: top: 200 nm; middle: 224 nm and bottom: 280 nm. From the chromatograms it is clear that BNP must be quantified at 200 nm, BIT at 254 nm and MI and CMI at 270 nm.

The four components are separated with more resolution than strictly necessary. A resolution of 1.5 means baseline separation and is usually enough for a correct integration of the chromatographic peak. On our chromatograms, the smallest resolution is between CMI and BIT, and equals almost 9. However, we must take into account that some matrix compounds may elute in the chromatogram and will interfere if the components are spaced too closely. Because the overall analysis time is not too excessive, we decided to use the proposed gradient.

3.4. Calibration

For routine analysis, an external calibration graph will be sufficient. A stock solution containing all four preservatives was prepared from the standards and ultra-pure water. This solution was diluted to give linear calibration graphs. The statistical data for these graphs are presented in **Table 5**. The 95% confidence limits and the LOD were calculated from the calibration according to Miller & Miller [34].

3.5. Assessing the Accuracy

The accuracy of the analysis in the real matrices was assessed using standard additions. This was done for each of the four preservatives, and each of the three different matrices provided. The exact composition of each of the matrices was not revealed to us, and we will refer to them as M1, M2 and M3. In standard addition, a known amount of standard is added to a sample, and the analytical

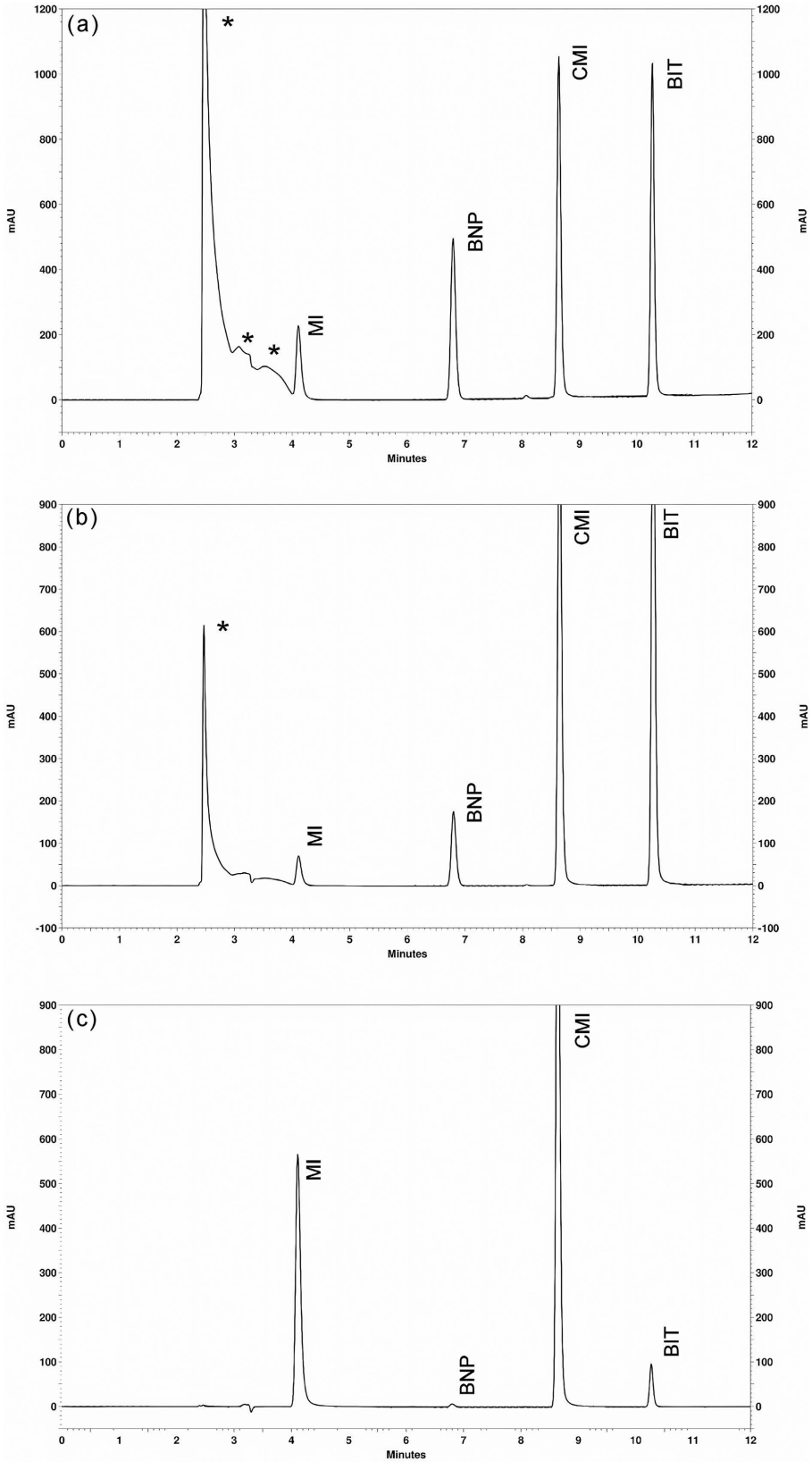


Figure 2. Sample chromatograms of the four preservatives at three different wavelengths. Peaks marked with * are due to the matrix of the preservatives. (a): detection wavelength 200 nm; (b): 224 nm and (c): 280 nm.

Table 4. Columns tested for the analysis.

Column	Column chemistry	Dimensions	Manufacturer	Comments
ODS Hypersil	C18	20 × 0.3 cm; 5 µm	Thermo Scientific	too much tailing
Xbridge Amide	HILIC	25 × 0.46 cm; 3.5 µm	Waters	matrix interference
Thermo Syncronis	HILIC	25 × 0.46 cm; 5 µm	Thermo Scientific	Coelution
Hypersil Gold CN	Nitrile	25 × 0.3 cm; 5 µm	Thermo Scientific	Coelution and matrix interference
Luna C18 (2)	C18	25 × 0.46 cm; 5 µm	Phenomenex	matrix interference
Allure biphenyl	Biphenyl	25 × 0.32 cm; 5 µm	Restek	Coelution and matrix interference
Hypersil Gold C18	C18	25 × 0.3 cm; 5 µm	Thermo Scientific	no interference, no coelution!

Table 5. Statistical data for the calibration curves.

Component	Slope([a])	Intercept([a])	r^2	linear range/ (ppm)([b])	LOD/ (ppm)([c])
MI	$(87.5 \pm 7.2) \times 10^4$	$(5.02 \pm 11.9) \times 10^3$	0.9998	5	0.062
BNP	$(21.4 \pm 2.7) \times 10^4$	$(1.5 \pm 2.1) \times 10^4$	0.9996	20	0.41
CMI	$(59.7 \pm 2.9) \times 10^4$	$(1.0 \pm 1.0) \times 10^4$	0.9999	10	0.076
BIT	$(16.6 \pm 2.2) \times 10^5$	$(2.9 \pm 3.3) \times 10^4$	0.9996	4	0.075

(a): ±95% confidence limit; (b): from 0 to indicated concentration in ppm; (c): Limit of detection.

signal is measured before and after the addition. The original concentration can now be calculated from the amount of standard added and the increase in signal. In practice, the original amount of component is calculated from the intercept on the x-(concentration) axis. The result thus calculated is compared to the result from the external calibration graph. For an accurate analysis, these two results should differ only slightly.

In our experiments, 4 different levels of standard were added to the original sample. Each point of the four levels was measured three times. Because all additions were weighted, small variations could occur in the mass of the original solution that was used for each of the additions. However, we took extreme care to weigh each time the same amount of original sample, so that these variations are certainly not larger than those that would be obtained with volumetric dilutions.

An example of such a standard addition curve is given in **Figure 3**.

This example is the addition curve for BNP in matrix M1. As can be seen, the linearity is excellent, so there is no need for correcting the different masses of the original sample using an internal standard. The original concentration can now be calculated from the negative intercept on the x-axis and compared to the result from the external calibration graph. The difference between the two is expressed as a percentage, and is taken as the bias, *b*. Because no certified reference materials for these analyses were available, we took the result from the standard addition as the more correct, or "true" value. The result from the external calibration graph follows the procedure that is used for the routine analysis and is

thus used as the "measured" value. The results are gathered in Table 6. As can be seen from these data, the accuracy is excellent and never surpasses 5%. Only for a few combinations of analyte and matrix it is in the range 3% to 4%.

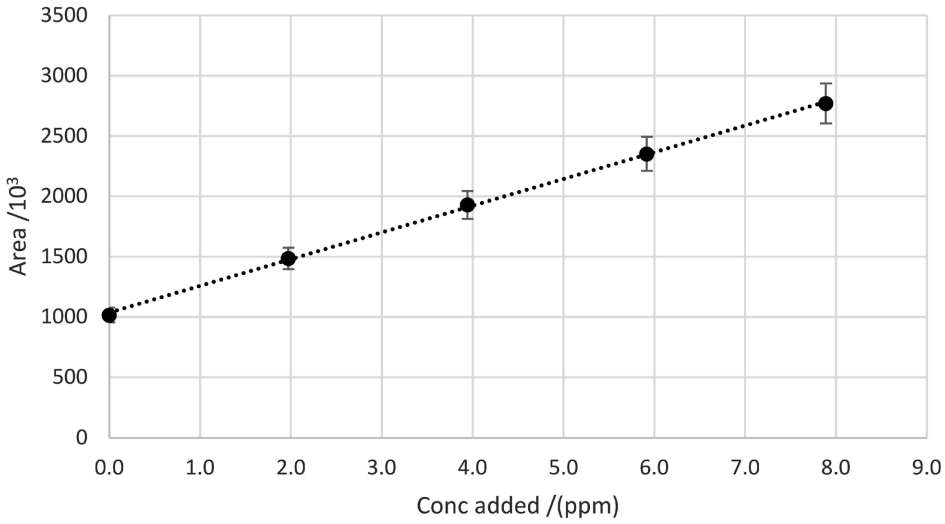


Figure 3. Standard addition curve for BNP in matrix M1. Each point is the average of three measurements. The erros bars reflect the 95% confidence limits.

Table 6. Comparison between standard addition and external calibration.

		Result from…						
Component	Matrix	Nominal concentration/ (ppm)	Dilution factor	Standard addition/ (ppm)	External calibration graph/(ppm)	Bias/(%)	%RSD([a])/%	U([b])/(%)
BNP	M1	250	25	232.5	232.1	−0.17	1.51	3.18
BNP	M2	250	25	297.2	299.4	0.74	1.39	3.52
BNP	M3	250	25	255.7	257.1	0.54	2.17	4.89
BIT	M1	75	25	69.4	70.1	0.99	1.95	4.88
BIT	M2	75	25	72.4	70.3	−2.90	1.89	6.67
BIT	M3	75	25	74.5	75.2	0.93	2.38	5.70
CMI	M1	15	6	11.8	11.4	−3.86	2.07	8.00
CMI	M2	15	6	11.1	10.7	−3.19	1.53	6.26
CMI	M3	15	6	10.8	10.4	−3.67	1.80	7.27
MI	M1	15	6	4.1	4.2	3.84	3.10	10.04
MI	M2	15	6	4.1	4.0	−1.41	1.22	3.85
MI	M3	15	6	3.9	3.9	0.19	2.08	4.35
MI	M1	75	25	75.9	76.8	1.26	1.88	5.01
MI	M2	75	25	80.9	80.1	−0.98	1.43	3.84
MI	M3	75	25	85.7	86.1	0.47	1.16	2.79

(a): Relative standard deviation of inter-day analyses, expressed as percentage, see Table 7; (b): *U*, Uncertainty, see section 3.6.

Table 7. Reproducibility and repeatability of the analysis.

	Matrix M1 BNP 250 ppm			Matrix M2 BNP 250 ppm			Matrix M3 BNP 250 ppm		
Day	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD
1	199.2	0.73	0.37	277.2	2.79	1.01	224.9	3.99	1.78
2	195.3	1.54	0.79	276.4	2.27	0.82	217.9	0.98	0.45
3	193.3	2.26	1.17	277.7	2.45	0.88	226.5	3.79	1.67
4	192.0	1.99	1.03	282.2	3.17	1.12	228.7	4.13	1.81
5	193.9	1.13	0.58	284.3	1.06	0.37	221.0	0.86	0.39
Interday([b])	194.8	2.93	1.51	279.6	3.89	1.39	223.8	4.86	2.17
	Matrix M1 BIT 75 ppm			Matrix M2 BIT 75 ppm			Matrix M3 BIT 75 ppm		
Day	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD
1	62.9	1.18	1.87	64.2	0.65	1.01	68.7	0.88	1.28
2	62.0	0.46	0.75	63.5	1.57	2.47	65.6	0.95	1.45
3	60.6	0.72	1.19	62.2	0.58	0.93	67.7	1.70	2.52
4	61.3	0.93	1.52	63.5	0.46	0.72	67.7	0.94	1.38
5	61.6	1.37	2.23	63.1	1.53	2.43	69.3	0.33	0.48
Interday([b])	61.7	1.20	1.95	63.3	1.19	1.89	67.8	1.62	2.38
	Matrix M1 CMI 15 ppm			Matrix M2 CMI 15 ppm			Matrix M3 CMI 15 ppm		
Day	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD
1	11.2	0.12	1.04	10.6	0.06	0.52	10.6	0.21	1.98
2	11.1	0.15	1.40	10.5	0.13	1.26	10.4	0.13	1.26
3	10.9	0.11	1.00	10.7	0.13	1.18	10.5	0.19	1.79
4	11.3	0.21	1.90	10.7	0.17	1.58	10.7	0.18	1.67
5	11.3	0.22	1.93	10.8	0.06	0.56	10.5	0.16	1.48
Interday([b])	11.2	0.23	2.07	10.7	0.16	1.53	10.5	0.19	1.80
	Matrix M1 MI 15 ppm			Matrix M2 MI 15 ppm			Matrix M3 MI 15 ppm		
Day	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD
1	4.2	0.11	2.50	3.9	0.02	0.63	3.9	0.03	0.86
2	4.2	0.06	1.49	3.9	0.05	1.27	3.9	0.07	1.91
3	4.0	0.05	1.24	4.0	0.04	0.90	3.9	0.07	1.68
4	4.3	0.13	3.12	4.0	0.07	1.73	4.0	0.12	3.06
5	4.3	0.11	2.63	4.0	0.05	1.19	3.9	0.05	1.30
Interday([b])	4.2	0.13	3.10	4.0	0.05	1.22	3.9	0.08	2.08
	Matrix M1 MI 75 ppm			Matrix M2 MI 75 ppm			Matrix M3 MI 75 ppm		
Day	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD	Mean([a])/(ppm)	s	%RSD
1	76.2	0.13	0.17	78.3	0.70	0.90	84.0	0.15	0.18
2	77.0	0.96	1.24	78.9	1.93	2.44	82.9	0.94	1.14
3	78.4	1.61	2.06	79.0	1.41	1.79	83.4	1.01	1.21
4	76.8	1.29	1.69	78.3	0.63	0.81	83.3	1.11	1.33
5	78.6	1.36	1.73	78.4	0.62	0.80	84.6	0.56	0.67
Interday([b])	77.4	1.45	1.88	78.6	1.12	1.43	83.6	0.97	1.16

(a): n = 5; (b): n = 5 for 5 consecutive days.

3.6. Repeatability and Reproducibility

For five consecutive days, five samples for each of the three matrices were prepared and analysed each day. The intra-day repeatability could thus be calculated as the Relative Standard Deviation, RSD (expressed as a percentage). The inter-day reproducibility was taken as the %RSD for all measurements over 5 consecutive days.

The results of the reproducibility experiments are presented in Table 7.

As can be seen from the data, the reproducibility is excellent: the intra-day repeatability is on the average less than two percent, and not much smaller than the inter-day reproducibility. This indicates that the proposed method of analysis is very precise and shows little variation from day to day.

3.7. Uncertainty

The uncertainty, *U*, is officially defined as the "*Quantity defining an interval about a result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand*" [32]. The uncertainty can be easily calculated from the bias and the reproducibility. The results of this calculation, for every matrix and analyte are compiled in Table 6.

With the exception of one combination of matrix and analyte, all analyses can be done with a total uncertainty of much less than 10%, which is an excellent result.

4. Conclusions

The aim of this research was to develop an analytical procedure for BNP, CMI, MIT and BIT that is fast, simple and reliable. With the proposed method the four preservatives can be analysed in 22 minutes with good precision and accuracy.

The sample preparation is very simple and fast: it is sufficient to dilute the sample with water and filtrate a portion for injection in the HPLC. The apparatus that is necessary for this analysis, a HPLC with gradient pump and DAD detector is quite simple and can be found in most laboratories.

Hence, we can conclude that the goals that were set at the onset of the project were met.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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