Analytical Methods

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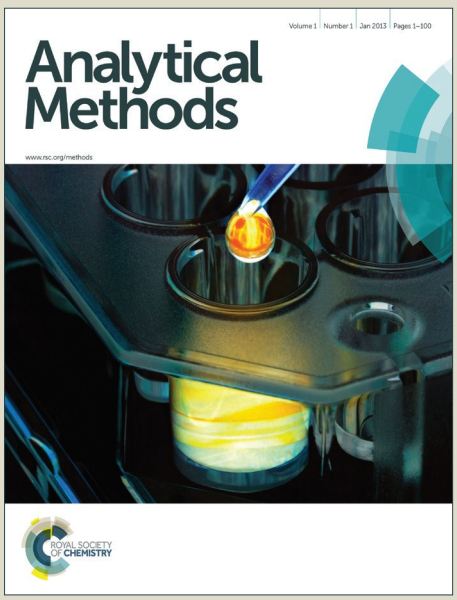

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ARTICLE

Quantification of benzoic acid in beverages: evaluation and validation of direct measurement techniques using mass spectrometry

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Orange juice is one of the most consumed beverages around the world and one of the most important export products in Brazil. Juice is a perishable good and industrial processing is generally made aiming at a greater lifetime for the product enabling long travels worldwide. One extremely common process for preservation is the addition of chemical preservatives such as benzoic acid. This addition has to be controlled in order to be kept at safe levels and not to give the product a bad taste. Many analytical methods are available for the analysis of this compound in beverages, however these methods tend to be laborious and long, involving tedious sample preparation steps. In this work, direct methods using mass spectrometry were evaluated for the rapid analysis of this compound. Two rapid methods were then developed: one using dilute and shoot preparation with flow injection analysis coupled to tandem mass spectrometric detection and another one using headspace sampling gas chromatography coupled to mass spectrometry with very little sample preparation, both using isotope dilution calibration. Both methods were validated and metrologically evaluated using certified reference materials and applied to real samples of different types of beverages: orange juice, soft drink, soy drink and orange flavored drink. Both methods presented excellent performance in all cases and were very fast to be performed, serving as references for laboratories which might want to implement these methods in their routine analyses.

Introduction

Orange juice is one of the most consumed beverages around the world. Data from the Brazilian Ministry of Development, Industry and Foreign Trade account for the daily export of 9.1 thousand tons in 2013, with a growing trend within the following years [1]. Since the juice might be subjected to long trips while being exported, it is very important that it can be stable during transportation and maintain its nutritive characteristics. These characteristics, however, also make it a good medium for bacterial growth, which turns it into a perishable good. In order to allow any type of commercial trade, industrial processing allows the juice to be preserved and last much longer. For this preservation, some compounds which prevent bacterial growth might be added, however these additives must have controlled addition at levels which are safe and also do not affect the taste of the product.

One of the additives used with this objective is benzoic acid, which is widely used in industry as a preservative, not only in orange juice. It has low toxicity in humans and is easily eliminated, being mainly excreted as hipuric acid in urine [2]. Excessive addition of benzoic acid might lead to loss of the flavor of the juice and hence cause unaccountable losses for the industry [3].

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The *Codex Alimentarius* establishes a limit for benzoic acid addition at 1000 mg.kg^{-1} for fruit juices [4], while FDA adopts the same limit for food in general [5]. Brazilian laws determine the same limit established by the *Codex Alimentarius*[6].

Benzoic acid has been used as a preservative in food for a long time and has even natural occurrence in some foods. Its antimicrobial activity is due to the non-ionized form, although benzoates can be used in order to improve water solubility. This non-ionized form is predominant in pH values of around 3 or lower and, for this reason, benzoic acid and benzoates are particularly adequate as preservatives for acid matrices as is the case of orange juice [7, 8]. Benzoic acid is also colorless and extremely cheap, characteristics which encourage its usage. For this reason, the main intake of benzoic acid by humans is in food. It is useful in inhibiting fungi and pathogenic bacterial growth in food [9].

Most of the analytical methods reported for the determination of benzoic acid in orange juice employ high performance liquid chromatography (HPLC) [10-12], but it is also possible to find methods which use gas chromatography with the flame ionization detector (GC-FID) [13, 14] and gas chromatography coupled to mass spectrometry (GC-MS) [15].

Modern analytical chemistry requires that analyses can be performed with minimum sample preparation and that instruments can be busy for the smallest time possible in order to allow high throughput monitoring of a different number of analytes. One of these rapid techniques is flow injection analysis (FIA), which consists of the injection of a sample which is carried by a pump in a carrier stream through a small

diameter tube before detection [16]. This technique, when coupled to a suitable detector might have a wide number of quantitative applications in different samples and analytes [17] [18]. The coupling of FIA with mass spectrometry (FIA-MS) is not a novelty and some applications can be found using single stage mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [19-21]. Another technique that might allow analyses with little sample preparation is the use of esterification reactions to create more volatile derivatives of the compounds of interest *in situ*, which has been described and successfully applied in other works [22], [23]. This might be used either for the determination of acids or alcohols, as the reaction consists of reacting an alcohol with a carboxylic acid, by acid catalysis, resulting in an ester of this acid which will then be analyzed, and water. After esterification, in many cases it is possible to analyze the volatile ester products of the analyte by static headspace sampling in gas chromatography (HSGC).

Both techniques allow the determination of compounds of interest in complex matrices with very little sample preparation, enabling the development of extremely fast but still reliable methods. Isotope dilution furnishes the best possible accuracy in this type of measurement since it enables the best correction possible for any effect that might occur to the analyte during sample preparation and analysis such as losses due to inefficient extractions or reactions, problems in chromatography and ionization in mass spectrometry. Since the physicochemical properties of the isotopologue are extremely similar to those of the analyte, we can assume that most of these effects will occur in a similar way to the analyte, thus leading to almost no variation in the analyte/internal standard ratio[24]. Thus the main goal of this work was to develop and validate methods for the quantification of benzoic acid in orange juice and similar products by mass spectrometry using FIA and the esterification followed by HS-GC in order to evaluate their performance as fast methods for this quantification.

Experimental

Chemicals and Reagents

The following chemicals and reagents were used: pure benzoic acid certified reference material from NIST (Gaithersburg, MD, USA), benzoic acid-D5 from Cambridge Isotope Laboratories (Tewksbury, MA, USA), methanol HPLC grade and sulfuric acid from Tedia (Farfield, OH, USA), certified reference material of benzoic acid in orange juice from HSA, with a reference value of 766 $mg.kg^{-1}$ ± 52 $mg.kg^{-1}$ (Outram Road, Singapore).

For the FIA-MS method, stock solutions of the analyte (benzoic acid) were prepared in methanol at a concentration of approximately 2500 $mg.kg^{-1}$ and stock solutions of internal standard (deuterated benzoic acid) were prepared in methanol at a concentration of approximately 1500 $mg.kg^{-1}$.

For the GC-MS method, stock solutions of the analyte were prepared in methanol at a concentration of approximately 8300 $mg.kg^{-1}$. An intermediate solution was prepared by dilution of this stock solution, in distilled water, to a concentration of approximately 1467 $mg.kg^{-1}$. A stock solution of internal standard were prepared in methanol at a concentration of approximately 28697 $mg.kg^{-1}$. An intermediate solution was prepared from dilution of this stock solution, in distilled water, to a concentration of approximately 761 $mg.kg^{-1}$.

All solutions were sealed and kept in the refrigerator at 4 °C until use. Solutions were prepared gravimetrically; this is, by weighing both the solutes and the solvents.

Sample preparation

FIA-MS quantification

An aliquot of approximately 1 g of each sample or calibration standard was blended approximately 1:1 with D5-benzoic acid solution, gravimetrically. An aliquot of 50 µL of these solutions was diluted with 1950 µL of methanol, filtered to 0.22 µm in a syringe filter and transferred to a 2.0 mL vial.

Headspace GC-MS Quantification

An aliquot of approximately 1 g of each sample or calibration standard was blended 1:1 with D5-benzoic acid solution, gravimetrically. 5 mL of distilled water were added to this mixture and 5 mL of the resulting solution were transferred to a 10 mL headspace vial. 1 mL of sulfuric acid and 0.5 mL of methanol were then added and the vial put in the headspace sampler.

Preparation of the calibration standards

FIA-MS Quantification

A calibration curve consisting of eight concentration levels (100-2400 $mg.kg^{-1}$) was prepared containing the analyte solution diluted with blank orange juice, and then prepared as stated in “sample preparation”.

Headspace GC-MS Quantification

A calibration curve consisting of seven concentration levels (100-2000 mg.kg-1) was prepared with the analyte solution diluted in distilled water and then prepared as stated in “sample preparation”.

FIA-MS conditions

FIA-MS analyses were performed in a Waters Acquity UPLC I-Class system coupled to a Xevo TQ mass spectrometer. The system was adapted to Flow Injection Analysis by connecting the exit of the injection valve directly to the electrospray probe with a 50 cm PEEK tube, with 1/16” of external diameter and 0.005” of internal diameter.

The carrier stream used was methanol/water (60:40) at a flow rate of 0.5 $mL.min^{-1}$. MS parameters: electrospray in negative mode, Capillary Voltage: 2.8 kV; Cone Voltage: 27 V; Desolvation Temperature: 300 °C; Desolvation Gas Flow: 650 $L.H^{-1}$; Mass spectrometric analysis was performed in Multiple Reaction Monitoring (MRM) mode for the transition 121>77 for the analyte and transition 126>82, for the internal standard, both with a collision energy of 10 eV. The injection volume was 1.0 µL and total run time was 0.5 minutes.

Headspace-GC-MS conditions

All analyses were performed in a Varian CP-3800 gas chromatograph coupled to a Saturn 2000 tridimensional ion trap mass spectrometer. A CombiPAL sampler was used for the headspace sampling.

The GC was equipped with a fused silica capillary column (CP SIL 5 MS, 30 m X 0.25 mm, 0.25 µm film thickness). Injection was performed at 210 °C in pulsed split mode with a ratio of 50:1 and a pressure pulse of 30 psi for 0.25 min. The injection volume was 0.5 mL. Helium was used as carrier gas with a flow rate of 1.0 mL/min. The GC oven was maintained at 100 °C for 5 minutes and then heated at a rate of 60 °C.min^{-1} until 280 °C. Headspace parameters were: incubation time: 40 minutes; incubation and syringe temperature: 90 °C, incubation velocity: 660 rpm. The MS was operated in electron ionization (EI) mode and the ion trap analyzer was set to a scan monitoring over the *m/z* range 45-200; Trap temperature: 200 °C; Manifold temperature: 80 °C; Transfer line temperature: 250 °C; Multiplier offset: -80 V.

Method Validation

Method validation was performed evaluating the following parameters for both methods: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity of the working range, repeatability, intermediate precision and bias.

Selectivity was assessed by the injection of matrix blank samples, spiked samples and standards in water in each method in order to determine if the expected peaks were absent in the blank (as well as interfering compounds) and present in the spiked samples and standards.

Linearity of the working range was evaluated by the construction of the calibration curves using linear least squares regression. Determination coefficient (r^2) greater than 0.99 was set as an acceptance criterion. Also, Cochran test was performed for the evaluation of the homogeneity of variances along the range (homocedasticity) and the residuals plot was evaluated to verify the absence of significant deviations from linearity.

Repeatability was verified by the injection of three replicate samples of the HSA CRM and the % relative standard deviation (% RSD) of these samples was used as the repeatability deviation. For intermediate precision, a set of 3 replicate samples of the same CRM was analyzed in 2 different days and their variances were compared by means of an F test. If the variances were equivalent, they were combined and the pooled % relative standard deviation was expressed as the intermediate precision deviation.

Bias evaluation was performed by analyzing the CRM from HSA in triplicate. The average result was compared to the certified value using the IRMM approach [25] and bias was also expressed numerically as the measurement result minus the certified value.

LOD and LOQ were determined by the signal to noise ratios. Spiked samples were prepared and analyzed until a signal to noise ratio of 3 was obtained which was considered the LOD. The same procedure was done to determine the LOQ with a signal to noise ratio of 10.

In order to evaluate the matrix effects in both methods, calibration curves were constructed in 6 different concentration levels for the HSGC-MS method and 8 levels for the FIA-MS method in both water and blank orange juice. The angular coefficients of these calibration curves were compared by means of a specific t test[26] in order to determine the statistical equivalency of both curves.

Measurement Uncertainty Estimation

For both methods, measurement equation is as follows (**Equation 1**):

$$w_x = \left(\frac{A_a}{A_{IS}} - b_0\right) \cdot \frac{m_{ISw} \cdot m_{ISs} \cdot P}{m_{ISf} \cdot b_1 \cdot m_s} \qquad (1)$$

Where: A_a is the analyte peak area; A_{IS} is the internal standard peak area; b_0 is the linear coefficient of the calibration curve; m_{ISw} is the mass of internal standard weighed for gravimetric preparation of internal standard solution; m_{ISf} is the final mass of the gravimetrically prepared internal standard solution; m_{ISs} is the mass of internal standard solution added to the sample; b_1 is the angular coefficient of the calibration curve; and m_s is the mass of sample used for analysis.

All measurement uncertainties were calculated by the uncertainty propagation approach as recommended by the ISO Guide for Uncertainty[27].

The first component of measurement uncertainty that had to be evaluated was the regression uncertainty, which was performed according to Hibbert[28]. Then the other components included in the estimation were the area ratio repeatability, the weighed mass of sample, weighed mass of internal standard solution and the purity of the analyte standard used in the calibration. Further details on the calculation approach used might be found in a previous work from our group[24].

Commercial beverage samples

In order to evaluate the performance of both methods in the measurement of benzoic acid in different types of drinks, some commercial beverages were analyzed. They comprised orange juices: Maguary, Shefa, Fruthos and Del Valle; One soy orange drink: Soy Suco; One orange soft drink: Fanta; Orange flavored non-gaseous drinks: Tampico, Lalita and Laranjinha.

Results and discussion

FIA-MS Method

The initial selectivity evaluation consisted of the acquisition of the four mass spectra presented in **Figure 1**. These are spectra for the blank matrix, benzoic acid in water, benzoic acid-D5 in water and a mix solution with both standards. Upon verification of spectral selectivity, further selectivity evaluation was performed by FIA analyses of a matrix without analyte and

a standard solution in water with benzoic acid and deuterated benzoic acid. These results are presented in **Figure 2**. The LOD was 30 mg.kg^{-1}, and the LOQ was 100 mg.kg^{-1}.

The calibration curve obtained for benzoic acid and its residuals plot are shown in **Figures 3** and **4**. The Cochran test demonstrated a homocedastic behavior for the calibration curve, and the residuals plot was free of trends. Thus, this calibration curve can be regarded as being in the linear working range for the method.

The certified reference material (CRM) from the Health Sciences Authority (HSA) with a certified value of (766 ± 52) mg.kg^{-1} was quantified in triplicate by the analytical curve in **Figure 3**. The measured value was 769 ± 15 mg.kg^{-1} and the relative standard deviation of the triplicate measurement was 0.65 %. For the intermediate precision a new set of triplicate samples was analyzed and the variances were compared to this first day variances in both days were equivalent and the pooled % RSD was 3.36 %. The bias evaluation was carried out statistically by the construction of confidence intervals for the measurement results. The results of the statistical evaluation show that there is no significant bias in the measured value within a 95 % confidence level.

Comparison of the curves in matrix and water led to a calculated t value of 0.03, while the critical t value (95 % probability) was 2.015, meaning that both curves are statistically equivalent and matrix effects are not significant.

Headspace GC-MS Method

Selectivity evaluation showed a detectable peak both for benzoic acid (*m/z* 105) and the internal standard (*m/z* 110) while none of these *m/z* ratios were observed in the blank matrix as shown in the chromatograms in **Figure 5**. The calibration curve obtained for benzoic acid and its residuals plot are shown in **figures 6 and 7**. The LOD was 0.7 mg.kg^{-1}, and LOQ 2.31 mg.kg^{-1}.

The same CRM from HSA was quantified in triplicate by the analytical curve in **Figure 6**. The measured value was 776.7 ± 7.5 mg.kg^{-1} and the relative standard deviation of the triplicate measurement was 0.48 %. For the intermediate precision the variances of the measurements done in two different days were evaluated by an F test and considered equivalent. The pooled % RSD for the intermediate precision was 1.15 %. The bias evaluation was carried out statistically by the construction of confidence intervals for the measurement results. The results of the statistical evaluation show that there is no significant bias in the measured value within a 95 % confidence level.

Comparison of the curves in matrix and water led to a calculated t value of 1.81, while the critical t value (95 % probability) was 2.04, meaning that both curves are statistically equivalent and matrix effects are not significant.

CRM bias evaluation

The last criterion evaluated for the performance of the methods was the determination of whether the measured results for a CRM are equivalent to the certified values. This evaluation is performed according to an approach described by Linsinger and coauthors [25, 29] and consists of a set of steps, the first being an evaluation of the absolute measurement bias (here called Δ_m) as shown in **Equation 2.**

$$\Delta_m = |C_{sample} - C_{CRM}| \qquad (2)$$

Where:
Δ_m = Absolute bias;
C_{sample} = Laboratory measurement result;
C_{CRM} = Certified value.

The second step in the evaluation is the combination of the measurement uncertainty with the uncertainty of the certified reference value as shown in **Equation 3.**

$$u_\Delta = \sqrt{u_{CRM}{}^2 + u_{sample}{}^2} \qquad (3)$$

u_Δ = Combined uncertainty between CRM and measured;
u_{CRM} = Uncertainty of the certified value;
u_{sample} = Uncertainty of the measurement in the laboratory for the CRM.

The calculated *uΔ* value is then expanded by a coverage factor of 2 to give the expanded combined uncertainty *UΔ*. The absolute bias must be then compared to *UΔ*. A *Δm* value smaller than *UΔ* indicates that the measured and the certified values are equivalent while *Δm* values greater than *UΔ* indicate a significant difference between the evaluated values. The results of this evaluation for both methods are shown in **Table 1**.

Table 1 - CRM comparison analyses. w = Mass fraction; *Uw* = standard uncertainty expanded; w_{CRM} = 766 mg/kg; U_{CRM} = 52 mg/kg.

Method	*w (mg/kg)*	U_w *(mg/kg)*	Δ_m	*UΔ*	*Bias (%)*
FIA-MS	769	15	3.14	54.1	0.41
GC-MS	776.7	7.5	10.7	52.6	1.40

Commercial beverage analyses

Some commercial beverages were analyzed using both developed methods. These analyses comprised several types of beverages similar to or containing orange juice. These were orange juices: Maguary, Shefa, Fruthos and Del Valle; Soy orange drink: Soy Suco; Orange soft drink: Fanta; Orange flavored non-gaseous drinks: Tampico, Lalita and Laranjinha. The measurement results for these samples are shown in **Table 2**.

In all analyses there is a trend for the HSGC-MS method to generate lower measurement uncertainty, both in the validation and in the commercial sample measurements. This

is probably due to the better repeatability obtained in the GC separation compared to the FIA analyses. This shall be considered when choosing the correct method to be used in each case: the FIA-MS method is much faster, but the measurement uncertainty with the HSGC-MS method is smaller. Both methods, however, involve very little sample preparation and will give very fast results. A triple-quadrupole electrospray mass spectrometer is expensive equipment and might be difficult for some laboratories to acquire. In light of the presented results, one might use the HSGC-MS method without any loss of performance and even gaining some advantage in terms of measurement uncertainty.

Table 2 - Measurement results by both mehods for different types of orange drinks.

Beverages	Type	GC-MS Method (mg/kg)	FIA-MS Method (mg/kg)
MAGUARY	Orange Juice	128.9 ± 8.2	112 ± 21
SHEFA	Orange Juice	<LOD	<LOD
FRUTHOS	Orange Juice	<LOD	<LOD
SOY SUCO	Soy Drink	<LOD	<LOD
DEL VALLE	Orange Juice	<LOD	<LOD
FANTA	Soft Drink	227.1 ± 9.3	234 ± 11
TAMPICO	Orange Flavored Drink	216 ± 10	216 ± 11
LALITA	Orange Flavored Drink	203 ± 10	209 ± 24
LARANJINHA	Orange Flavored Drink	426.1 ± 7.3	424.6 ± 9.0

Conclusion

Two methods were successfully developed, validated and compared for analyzing real samples. The FIA-MS method is extremely fast, since it uses a very simple sample preparation along with flow injection mass spectrometric analysis which takes 30 seconds per sample. The headspace GC-MS method has also has very little sample preparation and a relatively fast analysis in GC (8 minutes), However the headspace takes 40 minutes for preparing the sample for injection. This time can be minimized with a headspace sampler, in which many samples are stirred and heated while another sample is injected.

The quantitative analyses performed in this article shows that the methods are not only very fast, but also very reliable in the determination of benzoic acid in drinks.

The developed methods might be used by a different number of application laboratories with an ESI-MS/MS instrument or a HSGC-MS instrument, enabling laboratories which already have a structure for analyses to apply one of the methods without the need for large investment.

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Figure Captions:

Figure 1 - Full scan mass spectra obtained by direct infusion for selectivity evaluation.

Figure 2 - Response curves for FIA-MS. The x-axis is the time of analysis, in minutes.

Figure 3 - Calibration curve obtained for benzoic acid for FIA-MS quantification.

Figure 4 - Residuals plot of the calibration curve for FIA-MS quantification.

Figure 5 - Chromatograms for the HSGC-MS method of the analyte (m/z = 105) and internal standard (m/z = 110) in a spiked sample and a blank matrix.

Figure 6 - Calibration curve obtained for benzoic acid for HSGC-MS quantification.

Figure 7 - Residuals plot of the calibration curve for HSGC-MS quantification.

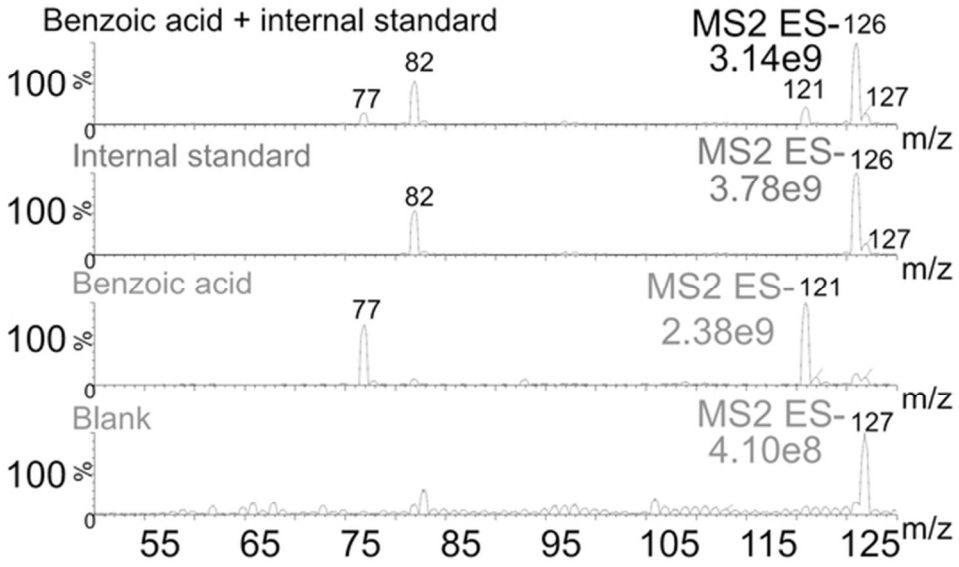

Figure 1 - Full scan mass spectra obtained by direct infusion for selectivity evaluation
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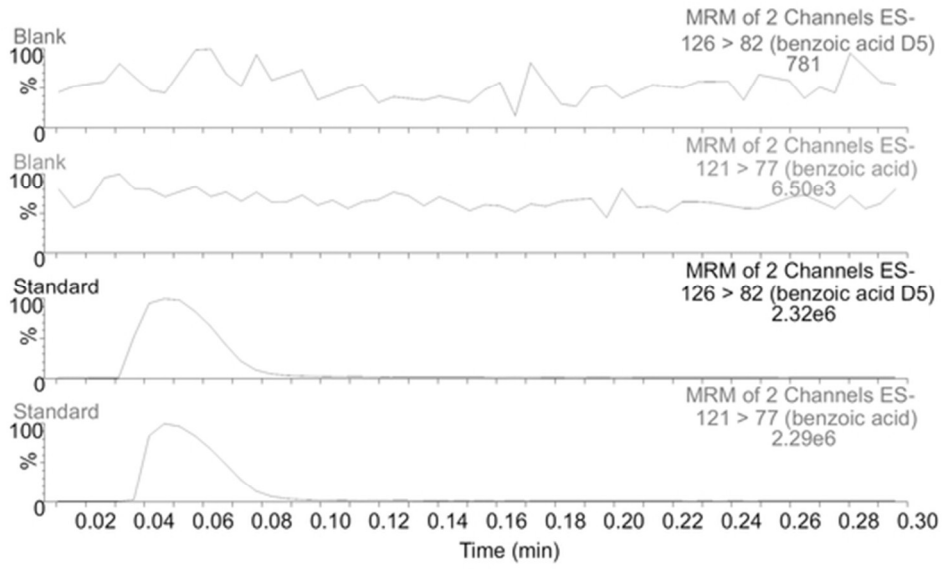

Figure 2: Response curves for FIA-MS. The x-axis is the time of analysis, in minutes.
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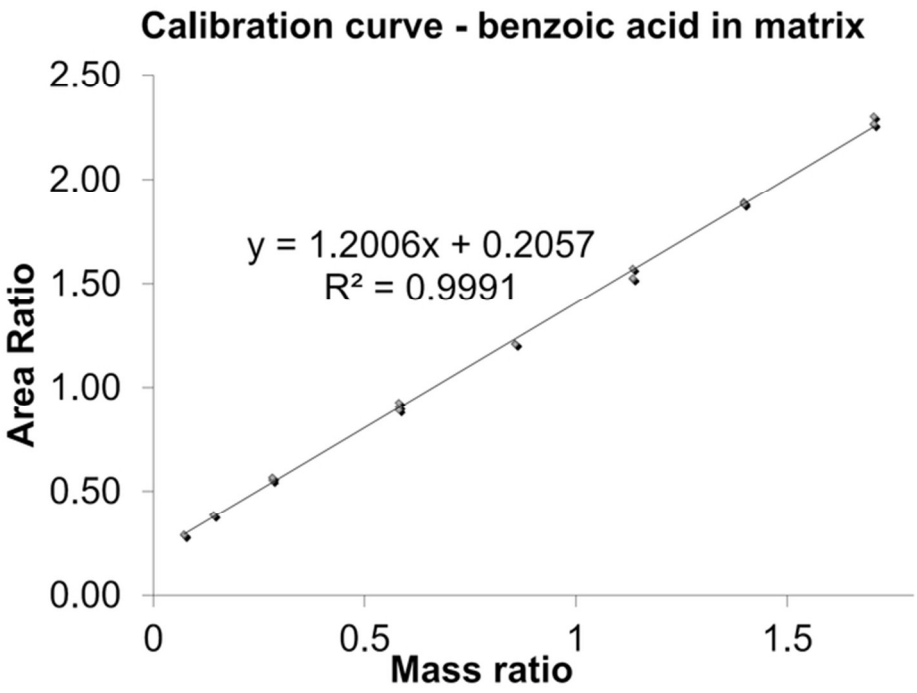

Figure 3 - Calibration curve obtained for benzoic acid for FIA-MS quantification.
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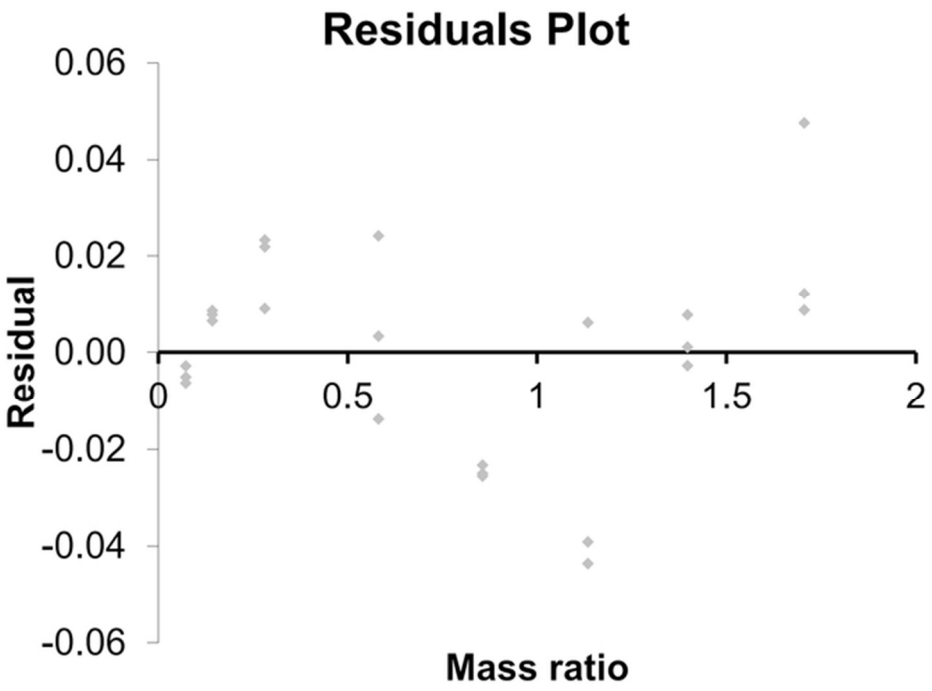


Figure 4 - Residuals plot of the calibration curve for FIA-MS quantification.
61x43mm (300 x 300 DPI)

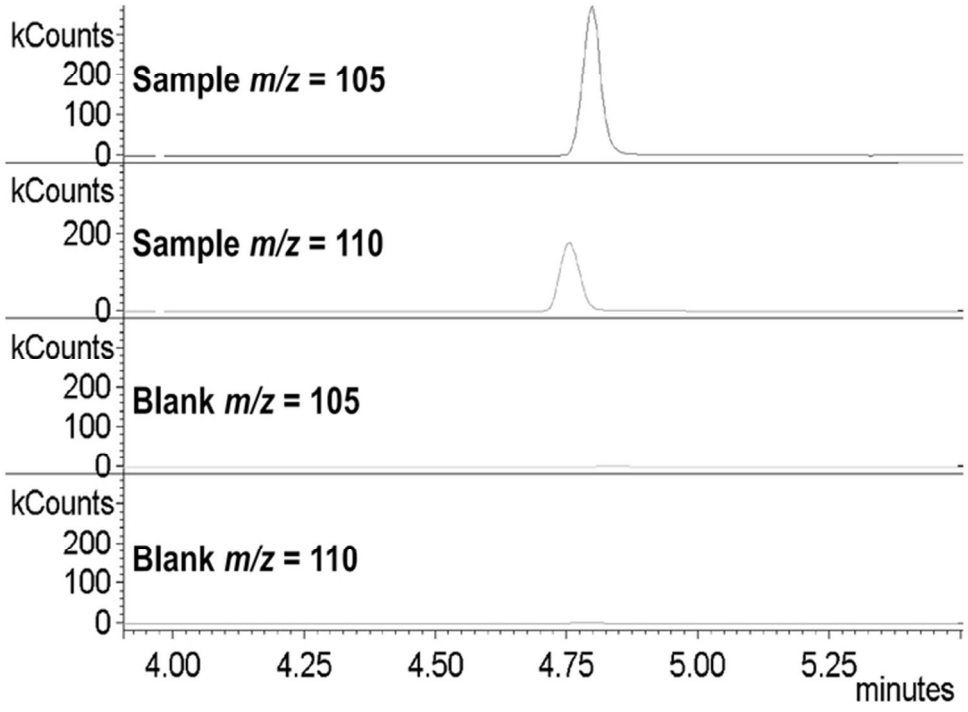


Figure 5 – Chromatograms for the HSGC-MS method of the analyte (m/z = 105) and internal standard (m/z = 110) in a spiked sample and a blank matrix.
64x48mm (300 x 300 DPI)

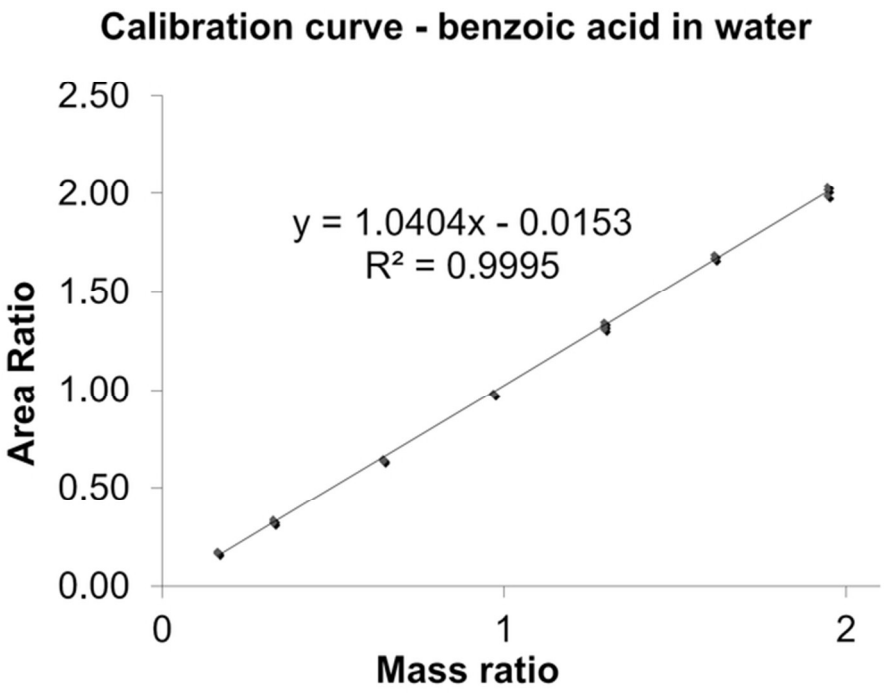


Figure 6 - Calibration curve obtained for benzoic acid for HSGC-MS quantification.
62x45mm (300 x 300 DPI)

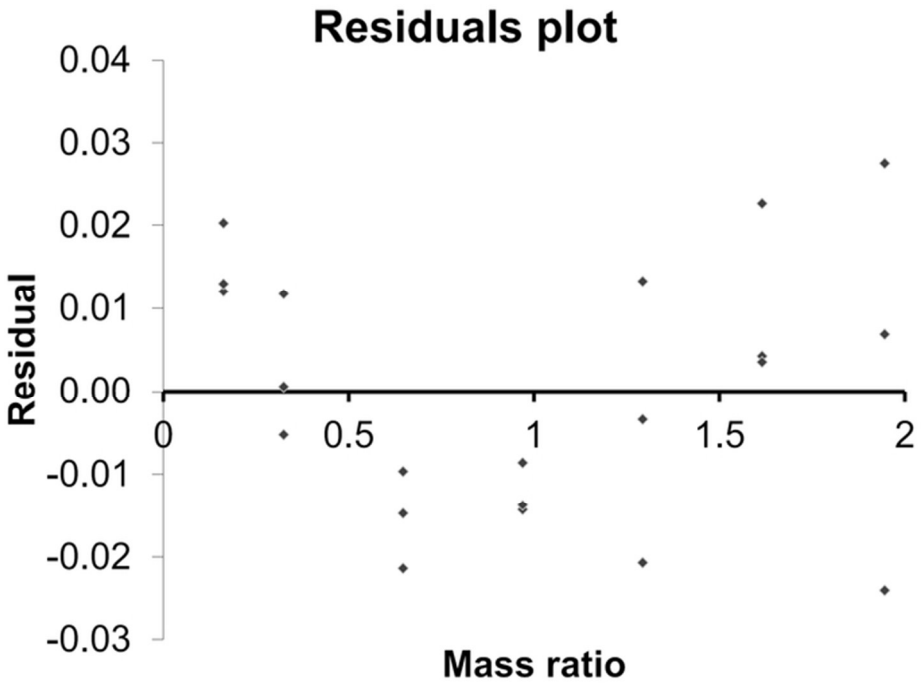
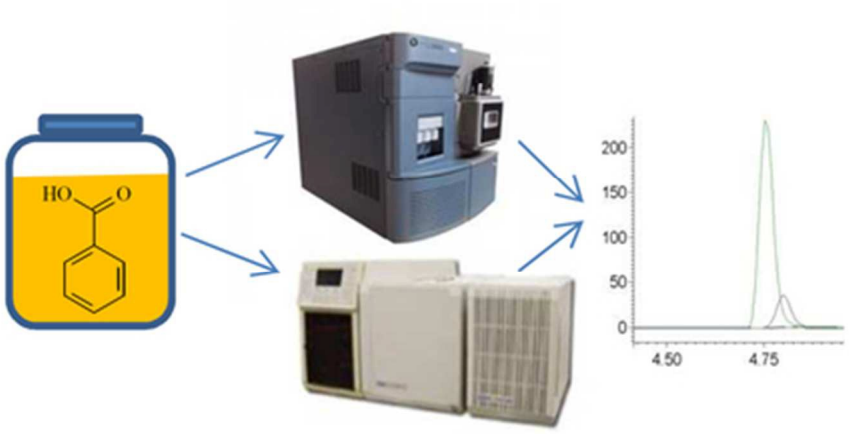


Figure 7 - Residuals plot of the calibration curve for HSGC-MS quantification.
62x46mm (300 x 300 DPI)



Two methods for the direct and rapid determination of benzoic acid in beverages were developed and compared.
39x19mm (300 x 300 DPI)