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Food Types:	Basmati rice

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# A comparison of calibration methods for quantification of Basmati and non-Basmati rice using microsatellite analysis

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## Abstract

The accurate quantification of non-Basmati rice in Basmati rice is central to the successful prosecution of adulteration, where non-basmati rice has been substituted for Basmati rice. The current method and three alternatives of constructing calibration curves for the measurement of non-Basmati rice in Basmati rice using microsatellite analysis were investigated. The methods compared involved power regression, linear regression (with and without  $\log_{10}$  transformation) and hyperbolic regression of the ratio of Basmati to non-Basmati peak areas. Assessments were made using the error uncertainty, standard error at the agreed limit of adulteration and 95% confidence intervals for five example data sets. The linear regression of the ratio of peak areas onto the ratio of content proportions was found to give the most precise calibration and thus enhanced quantification of the level of adulteration of Basmati rice with non-Basmati rice.

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3 **Key words:** microsatellite, Basmati, DNA, calibration  
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## 8 **Introduction**

9  
10 The consumption of Basmati rice has gained in popularity in recent years,  
11 particularly in the UK, which consumes 70% of the Basmati rice imported into  
12 the EU (approximately 155,000 tonnes/year). Its popularity stems from its  
13 unique characteristics, which include a popcorn-like fragrance and light fluffy  
14 grains on cooking. All rice varieties are closely related and only skilled  
15 technicians are able to use a variety of objective measures to distinguish varieties  
16 apart. However, varietal identification using DNA based analysis has been  
17 reported in the literature. The range of DNA based techniques includes random  
18 amplified polymorphic DNA (Ko et al 1994; Ohtsubo et al 1997) amplified  
19 fragment length polymorphisms (Mackill et al 1996) and microsatellite analysis  
20 (Bligh, et al 2000; Vemireddy et al 2007; Archak et al 2007). Of these  
21 techniques, microsatellite analysis appears to be the most successful and was  
22 recently used by the Food Standards Agency of the UK to survey the authenticity  
23 of Basmati rice on sale in the UK. The survey found that 17% of retail Basmati  
24 rice samples contained over 20% non-Basmati rice (Food Standards Agency,  
25 2004). The adventitious mixing of Basmati with non-Basmati rice can occur  
26 during the production of Basmati rice and hence up to 7% non-Basmati rice may  
27 be present legally in “Basmati rice” (agreed level of admixture in the UK Code  
28 of Practice). However the presence of more than 20% non-Basmati in some  
29 Basmati rice samples indicates wilful substitution, which if not declared would  
30 be illegal under food labelling rules. The accurate quantitation of the level of  
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3 non-Basmati rice is therefore of paramount importance for successful  
4 prosecution. However, microsatellite analysis is a multi-step process, the  
5 accuracy of which is affected by, and not limited to, the selection of the rice  
6 varieties as appropriate calibrants, the microsatellites used for analysis, the  
7 methodology used for DNA extraction, PCR and capillary electrophoresis, the  
8 efficiency of the software used for data collection and the methodology used for  
9 subsequent data analysis. We have focused on improving the methodology used  
10 to calculate the level of adulteration with non-Basmati rice. We report a  
11 comparison of the current method (construction of a standard curve with a best-  
12 fit parabolic line) and possible alternative methods of constructing calibration  
13 curves for the measurement of non-Basmati rice in Basmati rice using  
14 microsatellite analysis, to enable a more accurate measurement. Calibration  
15 methods were compared using a range of measures of uncertainty. The  
16 calibration method that was found to give the most precise calibration was the  
17 linear regression of the 'ratio of Basmati peak area to non-Basmati peak area'  
18 against the 'ratio of the proportion of Basmati rice to the proportion of non-  
19 Basmati rice', which when used together with the measurement of uncertainty,  
20 should provide enhanced quantification of the level of adulteration of Basmati  
21 rice with non-Basmati rice.  
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## 51 **Materials and methods**

### 52 *Sample DNA extraction and purification*

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54 Calibrants were prepared by gravimetrically mixing milled samples of Taraori  
55 (Basmati rice type) and Sherbati (long grain rice type). These samples were  
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3 further ground to a fine powder, in a food processor, prior to DNA  
4 extraction using the Nucleon<sup>®</sup> Phytopure kit (Amersham Pharmacia Biotech),  
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6 with the following minor modifications: the cooled cell lysate was centrifuged at  
7  
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9  
10 4500g for 15mins prior to transferring 0.5ml supernatant to a fresh  
11  
12 microcentrifuge tube and at the end of the procedure the DNA pellet was  
13  
14 resuspended in TE buffer which had been previously heated to 85°C.  
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### 21 *PCR assay conditions*

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23 Each PCR reaction (25µL), contained 1x Ampliqa Gold<sup>®</sup> reaction buffer, 0.625  
24  
25 Units Ampliqa Gold<sup>®</sup> DNA Polymerase (Applied Biosystems) and 0.5µM of  
26  
27 each deoxynucleotide triphosphate (Sigma) and 5 pmols of sense  
28  
29 (CTTAAATGGGCCACATGCG, 5' labelled with hexachlorofluorescein) and 5  
30  
31 pmols of anti-sense (TGCAAGAATCTGACCCGG) oligonucleotide primers  
32  
33 (Sigma) for rice microsatellite marker RM222. DNA template was diluted 1:4  
34  
35 DNA:water and 5µL added per reaction. Reactions were assembled in Axygen  
36  
37 thin walled PCR tubes and run on ABI 7900 PCR machine with the following  
38  
39 thermal cycling protocol: 94°C for 10 min followed by 45 cycles of 94°C for 1  
40  
41 min, 55°C for 1 min and 72°C for 1 min with a hold for 60min at 60 °C.  
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### 50 *Fluorescence gel electrophoresis*

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52 Amplicons were electrophoresed under denaturing conditions on an ABI377  
53  
54 (Applied Biosystems). Each reaction was run with a set of N,N,N',N'-  
55  
56 tetramethyl-6-carboxyrhodamine (TAMRA) labelled size standards (Applied  
57  
58 Biosystems). The results were analysed using GeneScan analysis software  
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(Applied Biosystems) and the total area, under each peak, for both alleles, at each calibration point, was calculated (Table 1).

### Calibration methods

Four calibration methods were compared for their ability to estimate accurate measurements of non-Basmati in Basmati rice. The error uncertainty, standard error at the agreed limit of adulteration and 95% confidence intervals were determined. The methods of calibration investigated were as follows:

#### *Power regression curve*

A frequently used method of constructing calibration curves, where a power regression curve is fitted to the ratio of Basmati peak area to non-Basmati peak area against the proportion of non-Basmati rice, is shown in equation 1.

$$r = ap^b \quad \text{Equation 1}$$

Where  $r$  is the ratio of 'Basmati peak area' to 'non-Basmati peak area',  $p$  is the proportion of non-Basmati rice and  $a$ ,  $b$  are constants estimated during model fitting.

#### *Linear regression of the ratio of peak areas onto the ratio of proportions*

An alternative option is to fit a linear regression to the ratio of Basmati peak area to non-Basmati peak area onto the ratio of the proportion of Basmati to the proportion of non-Basmati, as in equation 2.

$$r = cm + d \quad \text{Equation 2}$$

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3 Where  $m$  is the ratio of the proportion of Basmati rice to the proportion of non-  
4 Basmati rice, i.e.  $m = (1 - p) / p$  and  $c$ ,  $d$  are constants estimated during model  
5 fitting.  
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12 *Linear regression of the log of the ratio of peak areas onto the log of the ratio of*  
13 *proportions*  
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17 This method fits a linear regression to the log (base10) of the ratio of Basmati  
18 peak area to non-Basmati peak area against the log of the ratio of the  
19 proportion of Basmati to the proportion of non-Basmati, as in equation 3.  
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$$\log_{10}(r) = e \log_{10}(m) + f \quad \text{Equation 3}$$

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26 Where  $e$  and  $f$  are constants estimated during model fitting.  
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33 *Hyperbolic Regression Curve*  
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35 This alternative regresses the ratio of Basmati peak area to the non-Basmati  
36 peak area against the proportion of non-Basmati in the form of a hyperbolic line,  
37 as in equation 4. This is a generalized form of equation 2 that allows for the  
38 presence of an additional constant (Bligh, 2000).  
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$$r = g + h / (1 + kp) \quad \text{Equation 4}$$

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46 Where  $g$ ,  $h$  and  $k$  are constants estimated during model fitting.  
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## 54 **Statistical methods**

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56 Statistical analyses of five example calibration curves were performed by  
57 applying afore mentioned regression techniques to investigate which calibration  
58 method would provide the most accurate results for prediction. The uncertainty  
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3 associated with each calibration method was estimated by performing residual  
4 analyses (the residual is the difference between the observed response and its  
5 fitted model value) and by calculation of standard errors at the agreed limit of  
6 7% non-Basmati rice in Basmati rice, by method of inverse calibration (Brown,  
7 1993). The residual analyses consisted of comparing the residual standard  
8 deviation and a graphical inspection to test modelling assumptions. In addition,  
9 95% confidence intervals were calculated and the adjusted  $R^2$  statistic (the  
10 proportion of total variability in the response that is accounted for by the model,  
11 adjusted for degrees of freedom) was recorded. Appropriate transformations  
12 were performed for equation 3 to enable an appropriate comparison of errors  
13 and confidence widths to the other calibration methods. Without the  
14 transformation, measures may falsely appear smaller, as they are on the  
15 log(ratio of peak areas) scale rather than the ratio of peak areas scale. All  
16 analyses were performed using GenStat<sup>®</sup> 8.1 statistical software

## 37 38 **Results and discussion**

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41 Microsatellite analysis has now become the 'gold standard' method for the  
42 identification and quantification of long grain adulterant rice in Basmati rice  
43 following the survey conducted by the Food Standards Agency of the UK in  
44 2004 and the uptake of the methodology by analytical laboratories world wide  
45 (Archak et al 2007; Vemireddy et al., 2007). Rice varieties are closely related  
46 and no one marker can definitively identify a variety of rice, therefore, a panel of  
47 microsatellite markers are used to build a profile of the rice varieties present.  
48 Analysis of samples is usually a two step process, where the rice varieties of  
49 rice present is determined using up to 12 microsatellite markers, followed by  
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3 quantitative analysis, using either a single or occasionally, two microsatellite  
4 markers, appropriate for the constituents of the sample. The choice of  
5 microsatellite markers for both the identification and quantification stages are a  
6 matter of choice, although some, for example RM1, RM55 and RM44 are used  
7 by many laboratories (Food Standards Agency, 2004; Archak et al 2007). The  
8 work presented in this study uses the microsatellite marker RM222 for  
9 illustrative purposes, since this marker easily distinguishes between the two rice  
10 varieties used to construct the calibration curve: Taraori basmati and Sherbati  
11 long grain. However, the approach outlined in this manuscript has also been  
12 shown to be applicable for quantitation using the microsatellite markers M16,  
13 RM44, RM171 and RM201 and should in fact be appropriate for any  
14 microsatellite marker optimised for quantification.

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The data for the current study was generated by the analysis of multiple  
identical calibration curves, constructed from samples taken from single batches  
of Taraori Basmati and Sherbati long grain rice. In all 5 calibration curves were  
prepared and taken through the complete analysis, under repeatability  
conditions to provide independent, but linked datasets, enabling statistical  
analysis.

For illustration one of the five calibration data sets used to compare the  
calibration methods is shown in table 1. From this, the calculation of the ratio of  
peak areas and the ratio of proportions was determined.

Calibration models, in equations 1 to 4, applied to this data set are illustrated in  
figures 1 to 4. The changes in uncertainty between models are demonstrated  
by 95% confidence intervals for mean values. The linear regression of the ratio

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3 of peak areas onto the ratio of proportions (figure 2, equation 2) has the tightest  
4 intervals on the ratio of peak areas scale.  
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8 A visual check may lead the reader to believe that equation 3 (figure 3) has  
9 lesser scatter of points or tighter confidence intervals than the other equations.  
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11 However, the plots are on different scales (log(ratio of peak areas) for figure 3  
12 compared to of peak areas for figure 1, 2 and 4) and so the spread can not  
13 be crudely compared in this way. An investigation of the maximum confidence  
14 interval widths for each calibration model (table 2), after appropriate  
15 transformations for equation 3, show equation 1 can have over 15 times the  
16 confidence width of equations 2 and 4. Equation 3 can have up to 4 times that  
17 of equations 2 and 4. Therefore equations 2 and 4 give more accurate  
18 estimation of the ratio of peak areas.  
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31 Graphical residual checks highlighted a non-random structure for equation 1  
32 due to the poor fit of the model. Alternative model residuals were much  
33 improved with a random scatter of points around zero. This may be expected  
34 as the relationship between the ratio of peak areas against the ratio of  
35 proportions (equation 2) should be linear if the relative peak area for each type  
36 of rice is constant for each rice across the calibration range, or if relative peak  
37 areas are equal for each type of rice. In addition, the log of the peak  
38 areas against the log of ratio of proportions (equation 3) may be desirable as  
39 measurements based on PCR are cyclic in nature and therefore follow a  
40 lognormal distribution (assuming all other sources of error are minimal). Also,  
41 due to the reference concentration values often chosen, the log transformation  
42 can lead to a more evenly distributed set of calibration points.  
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3 Adjusted  $R^2$  values for each calibration data set for equations 2, 3 and 4 are  
4 mostly greater than 90%, where as the majority of adjusted  $R^2$  values of  
5 equation 1 are under 90% (table 3). Hence equation 1 explains less of the  
6 variation in the data sets than the alternatives and so has a poorer fit.  
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12 Residual standard deviation values for each calibration data set for equations 2  
13 and 4 are all less than 1.1 (table 4). Equations 1 and 3 can have over 5 and 2  
14 times the residual standard deviation of equations 2 and 4 respectively. Thus  
15 equations 2 and 4 provide better fits to the calibration data.  
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22 Standard error values at the agreed limit of 7% non-Basmati rice in Basmati rice  
23 show equation 1 to be consistently larger than the alternatives (table 5).  
24 Equation 2 has the smallest standard error for the first four calibrations and has  
25 the smallest maximum standard error overall. Hence, equation 2 performs  
26 more accurately when predicting at the limit of 7% non-Basmati rice.  
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34 An observation concerning the available data is the modest amount of  
35 information for the very low or very high percentage non-Basmati rice, where  
36 most assessments and predictions will be made. Therefore, it is advised that  
37 more reference materials at very high and very low concentrations are used for  
38 calibration.  
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46 In addition, when constructing calibration curves it is advised that the modelling  
47 checks that have been carried out during this investigation, such as graphical  
48 exploration of residuals, are continually performed. This enables monitoring of  
49 any underlying structure in the data which is not at first evident but which could  
50 lead to a poor fitting and so unreliable calibration curve being used for  
51 prediction of non-Basmati rice content.  
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## Conclusion

In conclusion, after investigation into current methods for constructing calibration curves for quantitative microsatellite analysis, it is recommended that a linear regression of the ratio of Basmati peak area to non-Basmati peak area onto the ratio of the proportion of Basmati to the proportion of non-Basmati (equation 2) is used in future calibrations.

It was found that equations 2 and 4 gave more accurate assessments when comparing error uncertainty and structure, standard errors at the agreed limit of 7% non-Basmati rice and 95% confidence intervals, when compared with equations 1 and 3. Overall, for these data sets, equation 2 was found to give the most precise calibration, as it reported the lowest standard error at the agreed limit of 7% non-Basmati on average. For future data sets of this kind, equations 2 and 4 may give very similar performances. However, due to ease of implementation in a non-statistical package, equation 2 is favoured.

Using this calibration method, and the measurement uncertainty derived from it, will provide improved quantification of the level of adulteration of Basmati rice with non-Basmati rice. Furthermore, this method can be utilised for other measurements taken from an electropherogram where the aim is to compare peak areas.

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**Table 1.** Total peak area under Basmati and Non-Basmati peaks for rice microsatellite marker RM222, from calibration data 1

Percentage of Non-Basmati	Ratio of proportions <sup>#</sup>	Basmati peak area	Non-Basmati peak area	Ratio of peak areas <sup>*</sup>
90	0.11	8573	107530	0.080
90	0.11	5338	77420	0.069
80	0.25	25523	117400	0.217
80	0.25	16418	76782	0.214
80	0.25	10755	49969	0.215
60	0.67	45955	62983	0.730
60	0.67	35775	48708	0.734
60	0.67	36784	49601	0.742
40	1.50	108668	71095	1.528
40	1.50	44688	26491	1.687
40	1.50	31717	18572	1.708
30	2.33	45393	17985	2.524
30	2.33	30896	11838	2.610
30	2.33	79124	31434	2.517
20	4.00	40269	5137	7.839
20	4.00	35717	4785	7.464
10	9.00	58007	5382	10.778
10	9.00	37721	3891	9.694
10	9.00	32834	3865	8.495
7	13.29	48012	3125	15.364
7	13.29	43202	2590	16.680
7	13.29	43736	3061	14.288

<sup>#</sup> Ratio of proportions = 'proportion of Basmati rice in sample' / 'proportion of non-Basmati rice in sample', e.g. 90% non-Basmati implies the ratio of proportions is 0.1/0.9=0.111

<sup>\*</sup> Ratio of peak areas = 'Basmati peak area' / 'Non-Basmati peak area'

**Table 2.** Maximum width of confidence intervals on ratio of peak areas scale (after transformation for equation 3 to enable comparability)

Calibration	Model			
	Equation 1	Equation 2	Equation 3	Equation 4
1	23.25	2.24	5.33	2.46
2	37.28	4.51	7.20	4.72
3	29.64	1.86	6.48	1.49
4	42.99	3.76	11.26	4.48
5	10.89	2.32	3.34	1.31

**Table 3** Adjusted R<sup>2</sup> values for calibration models

Calibration	Model			
	Equation 1	Equation 2	Equation 3	Equation 4
1	89.1	95.8	98.6	95.9
2	89.7	87.2	98.5	89.4
3	86.6	96.7	98.3	98.3
4	89.2	93.6	97.6	93.5
5	91.0	91.5	97.7	98.2

**Table 4.** Residual standard deviation for calibration models (after transformation for equation 3 to enable comparability)

Calibration	Model			
	Equation 1	Equation 2	Equation 3	Equation 4
1	3.529	1.018	1.468	1.052
2	6.033	1.080	2.457	1.068
3	4.629	1.048	1.839	1.029
4	6.106	1.022	2.297	1.048
5	1.113	1.054	1.291	1.043

**Table 5.** Standard error of the calibration models at the agreed limit of 7% non-Basmati rice in Basmati rice (after transformation for equation 3 to enable comparability)

Calibration	Model			
	Equation 1	Equation 2	Equation 3	Equation 4
1	0.0090	0.0023	0.0043	0.0032
2	0.0088	0.0043	0.0046	0.0077
3	0.0098	0.0020	0.0047	0.0025
4	0.0095	0.0030	0.0064	0.0046
5	0.0078	0.0032	0.0055	0.0006
Maximum	0.0098	0.0043	0.0064	0.0077



## Figures

Figure 1 Calibration of data set 1 using equation 1 - fitted power regression of the ratio of peak areas\* against the proportion of non-Basmati rice in the sample with 95% confidence intervals.

\* Ratio of peak areas = Basmati peak area / non-Basmati peak area

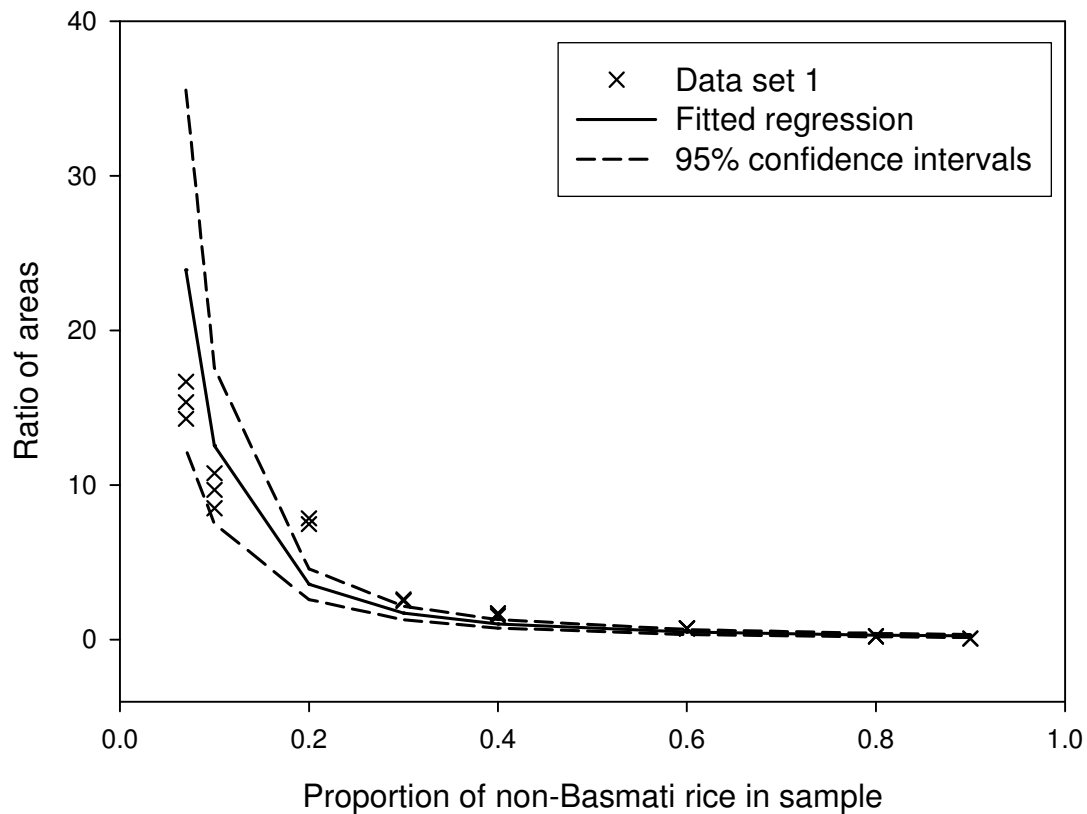


Figure 2 Calibration of data set 1 using equation 2 - fitted linear regression of the ratio of peak areas\* against the ratio of proportions# 95% confidence intervals.

\* Ratio of peak areas = Basmati peak area / non-Basmati peak area

# Ratio of proportions = proportion of Basmati rice / proportion of non-Basmati rice in sample

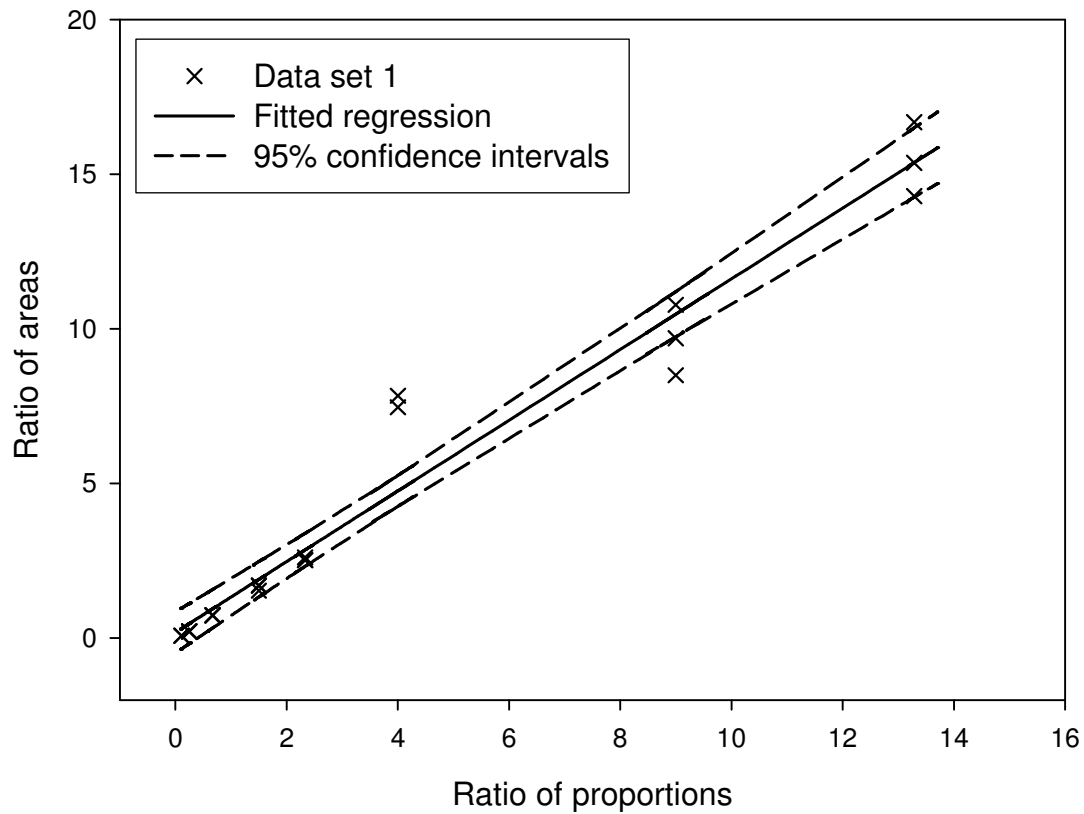


Figure 3 Calibration of data set 1 using equation 3 - fitted linear regression of the log (ratio of peak areas\*) against the log (ratio of proportions#) 95% confidence intervals.

\* Ratio of peak areas = Basmati peak area / non-Basmati peak area

# Ratio of proportions = proportion of Basmati rice / proportion of non-Basmati rice in sample

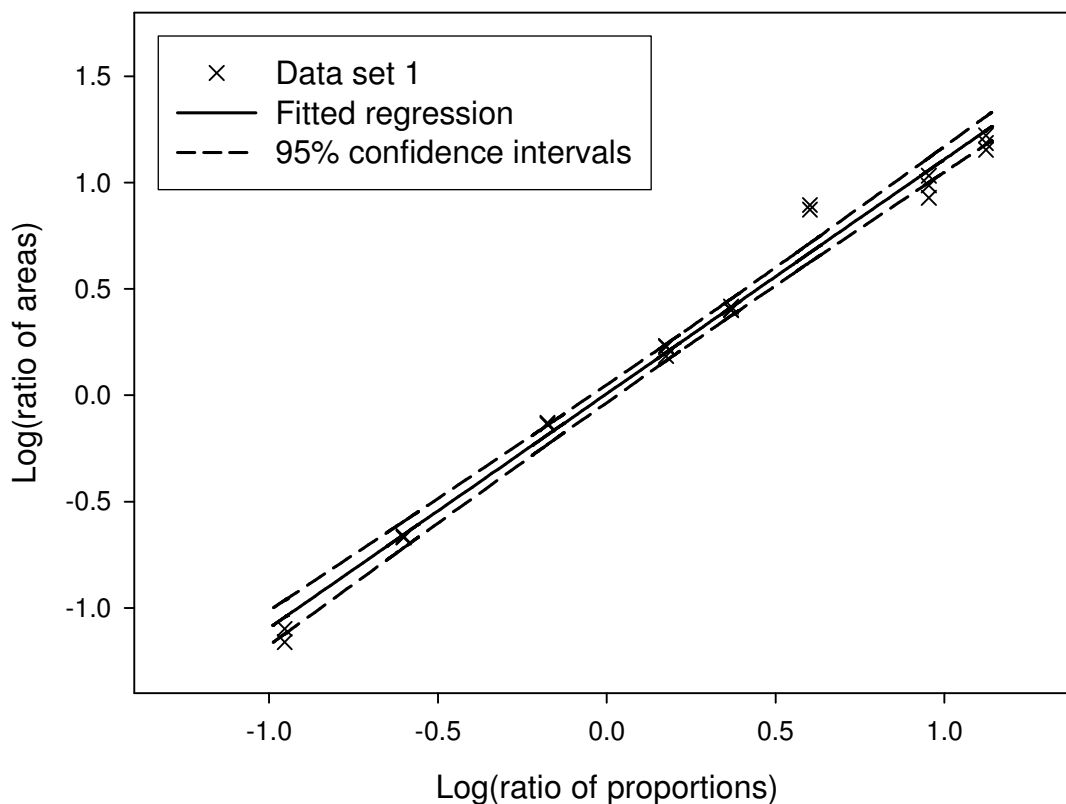


Figure 4 Calibration of data set 1 using equation 4 - fitted hyperbolic model of the ratio of peak areas\* against the proportion of non-Basmati rice in the sample (solid) 95% confidence intervals (dashed).

\* Ratio of peak areas = Basmati peak area / non-Basmati peak area

