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A Simple Confidence Band for the Michaelis-Menten Equation

Simon Brown[1], Noorzaid Muhamad[2], Kevin C Pedley[3], David C Simcock[3]

[1]School of Human Life Sciences, University of Tasmania, Locked Bag 1320, Launceston, Tasmania 7250, Australia
[2]Universiti Kuala Lumpur, Royal College of Medicine Perak, 3 Greentown Road, 30450 Ipoh, Perak, Malaysia
[3]Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand

Simon.Brown@utas.edu.au

Abstract. Analysis of enzyme kinetic data requires more than just comparisons of K_ms and V_{max}s using the corresponding error estimates of the parameters. This approach is often employed, but it can prompt contradictory and misleading inferences that might be avoided using a confidence band. We derive expressions for the confidence band for the Michaelis-Menten rate equation that rely on estimates and variances of K_m and V_{max}. These can be expressed in terms of the substrate concentration or the rate of reaction. While these equations are simple, they are nonlinear, which reinforces the need to consider both parameters simultaneously. The equations show that the amplitude of the confidence interval (ε) passes through a maximum if the variance of the K_m is sufficently large compared with the variance of V_{max}. We illustrate the value of the expressions by applying them to comparisons of the kinetics of enzymes involved in nitrogen metabolism in parasites. These examples confirm that (i) the variance of the estimate of the K_m has a particularly significant effect on ε and (ii) comparisons among K_ms and among V_{max}s are not necessarily sufficient to determine the significance of differences in activity.

Keywords: confidence band, glutamate dehydrogenase, glutamate synthase, Michaelis-Menten kinetics, parasite..

1. INTRODUCTION

One of the most common mathematical function in use by biologists is the Michaelis-Menten equation [1] which relates enzyme activity (v) to substrate concentration (s)

$$v = \frac{V_{max} s}{K_m + s}, \tag{1}$$

where V_{max} and K_m are kinetic constants [2]. Equation (1) is also employed in empirical models of a great variety of biological and physical processes [3, 4].

In reporting experimental data analysed using (1), it is usual to plot *v*, with some indication of error, against *s* and report V_{max} and K_m with their error estimates. Unfortunately, the data are often omitted and only V_{max} and K_m with their error estimates are reported (something we have done ourselves [5]) and sometimes even the latter are omitted. We have even seen reports in which several replicate estimates of V_{max} and K_m are provided without any attempt to merge them, prompting us to wonder which values should be taken to be reliable and just how similar they might be.

This has prompted us to consider how to use the error of the V_{max} (ε_V) and K_m (ε_K) to construct a confidence band for (1). Here we provide very simple expressions for the confidence band, examine some of the implications that arise from them and use them to analyse the kinetics of some enzymes involved in parasite nitrogen metabolism.

2. THEORY

The error of *v* (ε) can be estimated using

$$\varepsilon = \sqrt{\left(\frac{\partial v}{\partial V_{max}}\right)^2 \varepsilon_V^2 + \left(\frac{\partial v}{\partial K_m}\right)^2 \varepsilon_K^2}\ , \qquad (2)$$

[6], where

$$\frac{\partial v}{\partial V_{max}} = \frac{s}{K_m + s} = \frac{v}{V_{max}} \text{ and } \frac{\partial v}{\partial K_m} = \frac{-V_{max} s}{(K_m + s)^2} = \frac{-v}{K_m + s}.$$

Substituting these into (2) yields

$$\varepsilon = v\sqrt{\frac{\varepsilon_V^2}{V_{max}^2} + \frac{\varepsilon_K^2}{(K_m + s)^2}}\ , \qquad (3)$$

which can also be written as

$$\varepsilon = \frac{v}{V_{max}}\sqrt{\varepsilon_V^2 + \left(\frac{v}{s}\right)^2 \varepsilon_K^2} \qquad (4)$$

or, equivalently, as either

$$\varepsilon = \frac{v}{V_{max}}\sqrt{\varepsilon_V^2 + \frac{(v - V_{max})^2}{K_m^2} \varepsilon_K^2} \qquad (5)$$

or

$$\varepsilon = \frac{s}{K_m + s}\sqrt{\varepsilon_V^2 + \left(\frac{V_{max}}{K_m + s}\right)^2 \varepsilon_K^2}\ . \qquad (6)$$

Equations (3-6) are equivalent expressions for the estimated error of *v* (1).

3. RESULTS AND DISCUSSION

The confidence band obtained using (5) or (6) is illustrated in Figure 1A. It is apparent from Figure 1B (and (5-6)) that as v approaches V_{max} or s increases, the contribution of ε_K declines and ε approaches ε_V. However, at small s, where v is low, ε is also small (Figure 1A). At $s = K_m$, where $v = 0.5V_{max}$,

$$\varepsilon = \frac{1}{2}\sqrt{\varepsilon_V^2 + \frac{1}{4}\left(\frac{V_{max}}{K_m}\right)^2 \varepsilon_K^2},$$

and at large s, where $v \approx V_{max}$, ε might (or might not) decline slightly (Figure 2, A and B). An obvious dimensionless generalisation of this can be obtained from (6) by setting $s = \alpha K_m$

$$\frac{\varepsilon}{v} = \sqrt{\left(\frac{\varepsilon_V}{V_{max}}\right)^2 + \left(\frac{1}{1+\alpha}\right)^2\left(\frac{\varepsilon_K}{K_m}\right)^2}, \qquad (7)$$

in which case $v = (\alpha/(1+\alpha))V_{max}$ (1). The contributions of ε_K and ε_V reported in Figure 1B are estimated using

$$\phi_K = \frac{v\varepsilon_K}{K_m(1+\alpha)\varepsilon} \text{ and } \phi_V = \frac{v\varepsilon_V}{V_{max}\varepsilon},$$

respectively.

A maximum in the error (ε_{max}) occurs if

$$d = \frac{\varepsilon_K^2}{K_m^2} - 8\frac{\varepsilon_V^2}{V_{max}^2} > 0,$$

in which case ε_{max} occurs at positive values of s given by a root of a quadratic in s

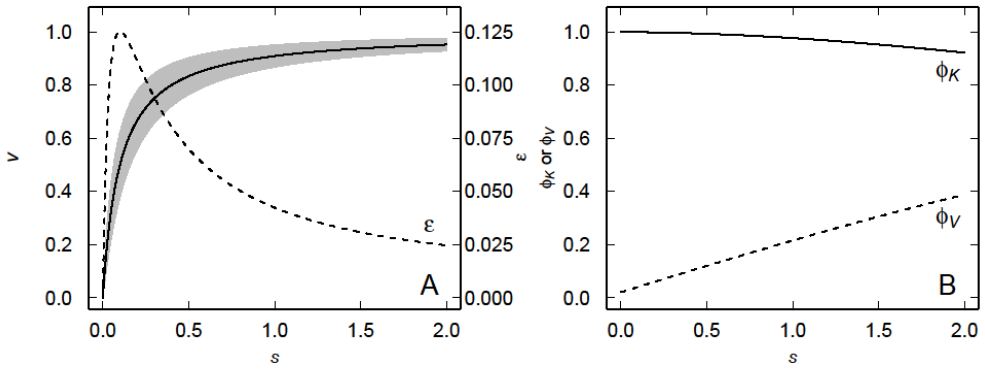

Figure 1. The Michaelis-Menten function (1) and the estimated confidence band (the grey zone around the solid line is ± ε, which is also shown) calculated using (5) (A) and the contribution of ε_V (ϕ_V) and ε_K (ϕ_K) to ε (B). It was assumed that $V_{max} = 1$, $K_m = 0.1$, $\varepsilon_V = 0.01$ and $\varepsilon_K = 0.05$.

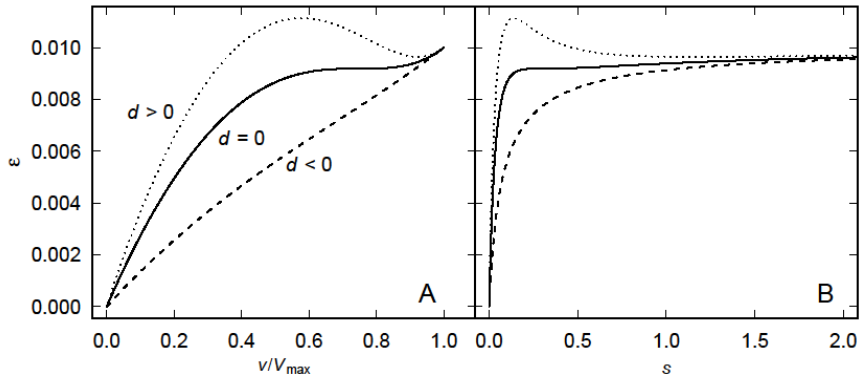

Figure 2. The estimated error of v (ε) as a function of (A) v (5) and (B) s (6). The three curves correspond to $d < 0$ (dashed curve, $\varepsilon_K = 0.001$), $d = 0$ (solid curve, $\varepsilon_K = 0.002828427$) and $d > 0$ (dotted curve, $\varepsilon_K = 0.0039$). It was assumed that $V_{max} = 1$, $K_m = 0.01$ and $\varepsilon_V = 0.01$.

$$K_m\left(K_m+s\right)^2\varepsilon_V^2+V_{\max}^2\left(K_m-s\right)\varepsilon_K^2=0$$

or

$$\left(1+\alpha\right)^2\frac{\varepsilon_V^2}{V_{\max}^2}+\left(1-\alpha\right)\frac{\varepsilon_K^2}{K_m^2}=0$$

from which it can be inferred that $\alpha > 1$ because

$$\frac{\left(1+\alpha\right)^2}{\alpha-1}=\frac{\varepsilon_K^2}{K_m^2}\frac{V_{\max}^2}{\varepsilon_V^2}, \qquad (8)$$

which must be positive, so any maximum can only occur at $s > K_m$. Writing the RHS of (8) as ρ, then the relevant root of (8) is

$$\alpha=\left\{\min\left(\frac{1}{2}\left(\rho-2\pm\sqrt{\rho\left(\rho-8\right)}\right)\right):\alpha>1,\rho>8\right\}.$$

Equivalently, but less usefully, positive values of v given by a root of the quadratic in v

$$2\varepsilon_K^2v^2-3\varepsilon_K^2V_{\max}v+\varepsilon_K^2V_{\max}^2+\varepsilon_V^2K_m^2=0$$

also provide estimates of the value of v at which ε_{max} occurs. This can be written as

$$2\beta^2-3\beta+1=-\frac{\varepsilon_V^2}{V_{\max}^2}\frac{K_m^2}{\varepsilon_K^2}<0$$

so $\beta < 1$, which is trivial if only because $\beta = v/V_{max} = \alpha/(1 + \alpha) < 1$. The value of ε_{max} can be determined by substituting the root obtained into (5) or (6). However, even where a maximum does not occur (as in the lower two curves in Figure 2A), ε is not linearly related to v.

4. RELATED WORK

Considerable effort has been expended in estimating the variances of the parameters of (1) [7-11], but none of these authors calculated the corresponding confidence band (5-6). The variation in the parameter estimates arises from experimental error and from the inherent heterogeneity of enzymes [12, 13]. Heterogeneity may arise from the distribution of the enzymes among different chemical states [13, 14] and from variation in the kinetic properties of the molecules [12], perhaps because of the distribution of molecular age, molecular crowding [14] or environment [15].

In most instances, replication can account for the experimental error involved in determinations of K_m and $V_{\max}$, which then prompts one to ask how to analyse the replicates [16, 17]. For example, where the parameters are measured for different enzyme preparations or in different reaction conditions it may be necessary to determine whether the parameter estimates are statistically different and, if appropriate, whether the estimates may be combined. These parameters are often estimated from linearised versions of (1), of which

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \frac{1}{s} + \frac{1}{V_{\max}} \tag{9}$$

[18] is in most common use, although there are several alternatives [19]. Unfortunately, linearising (1) introduces bias in the parameter estimates which are disproportionately influenced by the experimental error in data obtained at high and low substrate concentrations [20-22]. As nonlinear regression overcomes these difficulties and the necessary software is readily available, it is better to fit (1) directly to the data rather than employ (9) or other linearised forms [21]. The expression for the confidence band of any linear function, such as (9), is well known [6], but the corresponding expression for (1), given above (5-6), is not. One corollary of this is that the expression for the confidence band must be derived for each of the many variants of (1).

An important part of any regression analysis is the examination of the residuals [23] from which signs of systematic deviation may become apparent. While this analysis cannot be replaced by a confidence band, the latter does provide a convenient indication of the range of variation and it facilitates comparison of the results of regression analysis. Moreover, it is not possible to analyse the residuals where the raw data are not available, but expressions such as (5) and (6) can be applied using only available estimates of $V_{\max}$, ε_V, K_m and ε_K, as we show below.

5. APPLICATION TO EXPERIMENTAL DATA

As an example of the application of the analysis described above, we consider data relating to our long-standing interest in the nitrogen metabolism of parasites [5, 24, 25]. For clarity, we have summarised four examples in Table 1 (we refer interested readers to the original references for experimental conditions) and analyses of these

data are shown in Figure 3. These examples illustrate the value of (5) and (6) in the assessment of the significance of comparisons of estimates of the parameters in (1).

Table 1. Estimates of K_m and V_{max} obtained with the glutamate dehydrogenases (GDHs) of *Trichomonas vaginalis* [26], *Dirofilaria immitis* [27] and *Teladorsagia circumcincta* [25, 28], and the glutamate synthase (GS) of *T. circumcincta* [29].

Species/Enzyme	Conditions	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1} protein)	Figure
T. vaginalis/GDH[a]	reductive (αKG)	0.6 ± 0.3	144 ± 72	3A
	oxidative (glu)	1.2 ± 0.4	50 ± 27	3A
D. immitis/*m*GDH[b]	0 mM ATP	0.4 ± 0.2	6.8 ± 0.6	3B
	0.25 mM ATP	2.1 ± 0.9	13.5 ± 2.6	3B
T. circumcincta/GDH[b]	recombinant	0.05 ± 0.01	1280± 34	3C
	homogenate	0.025± 0.004	327 ± 8	3C
T. circumcincta/GS[b]	larval	0.6 ± 0.05	37 ± 4.5	3D
	adult	1.5±0.15	65 ± 9.5	3D

[a] Errors are ± SD (n = 15)
[b] Errors are ± SEM

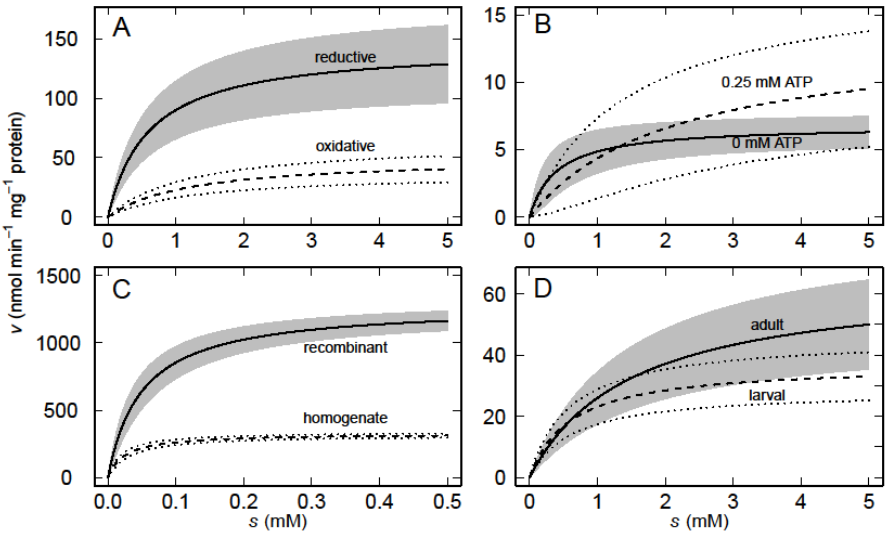

Figure 3. Activities of GDH (A-C) and GS (D) as a function of substrate concentration based on the K_m and V_{max}s, and their associated errors, summarised in Table 1. In each panel the 'control' (solid curve) and 'test' (dashed curve) curved are calculated using (1) and the values in Table 1. The 95% confidence intervals ('control': grey band; 'test': delimited by dotted lines) were calculated using (6).

Glutamate dehydrogenase (GDH, E.C. 1.4.1.3) catalyses the reversible oxidative deamination of glutamate (glu) to α-ketoglutarate (αKG). Turner and Lushbaugh [26] suggested that the rate of oxidative deamination differed from the rate of reductive amination in the protozoan parasite *Trichomonas vaginalis*. Their suggestion was based on significant differences between (i) the K_ms for glu and αKG and (ii) the V_{max}s of the reactions (Table 1). It is clear from Figure 3A that the authors were correct in their assertion.

Some parasite GDHs, like the mammalian enzymes, are allosterically modified by nucleotides [30]. Turner et al. [27] suggested that *Dirofilaria immitis* has two isozymes, a mitochondrial GDH (*m*GDH) and a cytoplasmic form. Without any specific statistical test they argued that the presence of 0.25 mM ATP increased the K_m and the V_{max} of the *m*GDH (Table 1). This prompts the question as to whether there is a significant effect on the activity of the enzyme. It is apparent from Figure 3B that there is little evidence for any significant effect of ATP on the enzyme activity.

We have characterised the GDH from *Teldorsagia circumcincta* [25], a nematode parasite, and the kinetic properties of the recombinant enzyme have also been characterised [28]. Unsurprisingly, the V_{max} of the recombinant enzyme is greater than that of the crude enzyme, but the reported K_ms are not different (Table 1). Naturally, we wish to know whether there is a significant difference in the activities of the two enzymes. It is clear from Figure 3C that there is significantly different activity between the homogenate and recombinant GDH, as one would have anticipated. The most surprising feature of the recombinant enzyme is that the V_{max} is so low. Skuce et al. [31] reported a V_{max} of 718 μmol min^{-1} mg^{-1} protein for the purified enzyme from the closely related nematode *Haemonchus contortus*, and Rhodes and Ferguson [32] reported a V_{max} of 3.38 μmol min^{-1} mg^{-1} protein for a partially purified *H. contortus* GDH. It is remarkable that the V_{max} of the recombinant *T. circumcincta* GDH is only about four times that of the unpurified enzyme (Table 1) and is no more than a third of that of partially purified *H.* contortus GDH [31, 32]. The inference we draw from this observation is that the reported V_{max} is an underestimate (by a factor of about 100) or that the addition of the six-his tag, the expression of the enzyme in *Escherichia coli* or the purification somehow inactivated the enzyme.

Glutamate synthase (GS, E. C. 1.4.1.14) catalyses the formation of glutamate from glutamine and α-ketoglutarate. Kinetic characteristics of GS from the larval and adult stages of *T. circumcincta* have been reported [29]. The data prompted us to ask whether the kinetic properties of the enzyme differ between lifecycle stages. A simple comparison of the V_{max}s and K_ms for glutamine (Table 1) might prompt the tentative conclusion that the V_{max}s might not be different, but the K_ms might differ. However, the 95% confidence band prompts the conclusion that there is no evidence for a significant difference in the activity of the enzyme between the two stages (Figure 3D).

These four examples (Table 1 and Figure 3) illustrate two important results. First, the ε_K/K_m ratio makes an especially significant contribution to ε (Figure 1B) and if this ratio is large its influence may be apparent even when s is many times K_m

(Figure 3B). The corollary of this, well known to practising enzymologists, is that a small ε_K/K_m ratio is, generally, more desirable than a small $\varepsilon_V/V_{\max}$ ratio (compare Figures 3A and 3B), as is clear from (7). Of course it is desirable that both ratios are small (as in Figure 3C), but there is cause for concern where both are large (Figure 3D). Second, comparisons among K_ms and among $V_{\max}$s are not necessarily sufficient to determine the significance of differences in activity. Large differences in $V_{\max}$ (Figure 3C) can moderate the effects of a large ε_K/K_m ratio.

6. CONCLUSIONS

Successful comparisons of the kinetic properties of enzymes requires more than a statistical test of the difference between the reported K_ms and $V_{\max}$s. The simple expressions for a confidence band (3-7) for the Michaelis-Menten expression provide a basis for making such comparisons.

REFERENCES

1. Michaelis, L and Menten, ML, "Die Kinetik der Invertinwirkung", Biochemische Zeitschrift 1913; 49: 333-369.
2. Briggs, GE and Haldane, JBS, "A note on the kinetics of enzyme action", Biochemical Journal 1925; 19: 338-339.
3. López, S, France, J, Gerrits, WJJ, Dhanoa, MS, Humphries, DJ and Dijkstra, J, "A generalized Michaelis-Menten equation for the analysis of growth", Journal of Animal Science 2000; 78: 1816-1828.
4. Tjørve, E, "Shapes and functions of species-area curves: a review of possible models", Journal of Biogeography 2003; 30: 827-835.
5. Brown, S, Muhamad, N and Simcock, DC, "Estimating enzyme kinetic parameters from apparent KMs and Vmaxs", International Journal of Chemical and Biological Engineering 2010; 3: 200-205.
6. Dudewicz, EJ and Mishra, SN, "Modern mathematical statistics", John Wiley and Sons, Inc., New York. 1988.
7. Achcar, JA, de Cássia Favoretti, A and Mazucheli, J, "Approximate Baysian analysis for the Michaelis-Menten model", Journal of the Chilean Statistical Society 1996; 13: 1-23.
8. Cornish-Bowden, A and Eisenthal, R, "Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods", Biochemical Journal 1974; 139: 721-730.
9. Cornish-Bowden, A and Wong, JT-F, "Evaluation of rate constants for enzyme-catalysed reactions by the jacknife technique. Application to liver alcohol dehydrogenase", Biochemical Journal 1978; 175: 969-976.
10. Jones, ME, "Analysis of algebraic weighted least-squares estimators for enzyme parameters", Biochemical Journal 1992; 288: 533-538.
11. Murphy, EF, Gilmour, SG and Crabbe, MJC, "Efficient and accurate experimental design for enzyme kinetics: Bayesian studies reveal a systematic approach", Journal of Biochemical and Biophysical Methods 2003; 55: 155-178.

12. Steinbach, PJ, Chu, K, Frauenfelder, H, Johnson, JB, Lamb, DC, Nienhaus, GU, Sauke, TB and Young, RD, "Determination of rate distributions from kinetic experiments", Biophysical Journal 1992; 61: 235-245.
13. Brown, S, "Estimating the distribution of forms of cytochrome oxidase from the kinetics of cyanide binding", ICFAI Journal of Biotechnology 2008; 2: 51-60.
14. Minton, AP and Wilf, J, "Effect of macromolecular crowing upon the structure and function of an enzyme: glyceraldehyde-3-phosphate dehydrogenase", Biochemistry 1981; 20: 4821-4826.
15. Kuthan, H, "Self-organisation and orderly processes by individual protein complexes in the bacterial cell", Progress in Biophysics and Molecular Biology 2001; 75: 1-17.
16. Duggleby, RG, "Pooling and comparing estimates from several experiments of a Michaelis constant for an enzyme", Analytical Biochemistry 1990; 189: 84-87.
17. Phillips, GR, Harris, JM and Eyring, EM, "Treatment of replicate measurements in kinetic analysis", Analytical Chemistry 1982; 54: 2053-2056.
18. Lineweaver, H and Burk, D, "The determination of enzyme dissociation constants", Journal of the American Chemical Society 1934; 56: 658-666.
19. Dowd, JE and Riggs, DS, "A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations", Journal of Biological Chemistry 1965; 240: 863-869.
20. Blunck, M and Mommsen, TP, "Systematic errors in fitting linear transformations of the Michaelis-Menten equation", Biometrika 1978; 65: 363-368.
21. Johnson, ML, "Why, when, and how biochemists should use least squares", Analytical Biochemistry 1992; 206: 215-225.
22. Gains, N, "Unreliability of rate constants derived from a linear transformation of kinetic data, with special reference to cholesterol equilibration between phospholipid vesicles", Biochemical Journal 1992; 283: 537-539.
23. Cook, RD and Weisberg, S, "Residuals and influence in regression", Chapman and Hall, New York. 1982.
24. Muhamad, N, Brown, S, Pedley, KC and Simpson, HV, "Kinetics of glutamate dehydrogenase from L3 Ostertagia circumcincta", New Zealand Journal of Zoology 2004; 31: 97.
25. Muhamad, N, Simcock, DC, Pedley, KC, Simpson, HV and Brown, S, "The kinetics of glutamate dehydrogenase of Teladorsagia circumcincta and the lifestyle of the parasite", Comparative Biochemistry and Physiology 2011; 159B: 71-77.
26. Turner, AC and Lushbaugh, WB, "Trichomonas vaginalis: characterization of its glutamate dehydrogenase", Experimental Parasitology 1988; 67: 47-53.
27. Turner, AC, Lushbaugh, WB and Hutchison, WF, "Dirofilaria immitis: comparison of cytosolic and mitochondrial glutamate dehydrogenases", Experimental Parasitology 1986; 61: 176-183.
28. Umair, S, Knight, JS, Patchett, ML, Bland, RJ and Simpson, HV, "Molecular and biochemical characterisation of a Teladorsagia circumcincta glutamate dehydrogenase", Experimental Parasitology 2011; 129: 240-246.
29. Umair, S, Bland, RJ and Simpson, HV, "Glutamate synthase, but not GABA shunt enzymes, contributes to nitrogen metabolism of the sheep abomasal nematode parasites Haemonchus contortus and Teladorsagia circumcincta", Experimental Parasitology 2011; 127: 9-13.
30. Hudson, RC and Daniel, RM, "L-Glutamate dehydrogenases: distribution, properties and mechanism", Comparative Biochemistry and Physiology 1993; 106B: 767-792.
31. Skuce, PJ, Stewart, EM, Smith, WD and Knox, DP, "Cloning and characterization of glutamate dehydrogenase (GDH) from the gut of Haemonchus contortus", Parasitology 1999; 118: 297-304.
32. Rhodes, MB and Ferguson, DL, "Haemonchus contortus: enzymes. III. Glutamate dehydrogenase." Experimental Parasitology 1973; 34: 100-110.