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Short communication

Enzymatic analysis of α-ketoglutaramate—A biomarker for hyperammonemia

Lenka Halámková [a], Shay Mailloux [a], Jan Halámek [a], Arthur J.L. Cooper [b,*], Evgeny Katz [a,**]

[a] Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY 13699, USA
[b] Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

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ABSTRACT

Two enzymatic assays were developed for the analysis of α-ketoglutaramate (KGM)—an important biomarker of hepatic encephalopathy and other hyperammonemic diseases. In both procedures, KGM is first converted to α-ketoglutarate (KTG) via a reaction catalyzed by ω-amidase (AMD). In the first procedure, KTG generated in the AMD reaction initiates a biocatalytic cascade in which the concerted action of alanine transaminase and lactate dehydrogenase results in the oxidation of NADH. In the second procedure, KTG generated from KGM is reductively aminated, with the concomitant oxidation of NADH, in a reaction catalyzed by L-glutamic dehydrogenase. In both assays, the decrease in optical absorbance ($\lambda = 340$ nm) corresponding to NADH oxidation is used to quantify concentrations of KGM. The two analytical procedures were applied to 50% (v/v) human serum diluted with aqueous solutions containing the assay components and spiked with concentrations of KGM estimated to be present in normal human plasma and in plasma from hyperammonemic patients. Since KTG is the product of AMD-catalyzed hydrolysis of KGM, in a separate study, this compound was used as a surrogate for KGM. Statistical analyses of samples mimicking the concentration of KGM assumed to be present in normal and pathological concentration ranges were performed. Both enzymatic assays for KGM were confirmed to discriminate between the predicted normal and pathophysiological concentrations of the analyte. The present study is the first step toward the development of a clinically useful probe for KGM analysis in biological fluids.

1. Introduction

Hyperammonemia is a major factor contributing to the encephalopathy associated with acute and chronic liver disease [1–5] and with many other diseases, including inborn errors of the urea cycle [1,4]. However, blood and cerebrospinal fluid (CSF) levels of ammonia may fluctuate considerably in patients with hyperammonemic encephalopathy and may not coincide with degree of encephalopathy. For example, in a recent case study of a patient who overdosed on acetaminophen blood ammonia levels peaked before the onset of neurological symptoms and approached normal during a time when the patient was deeply comatose [6].

Ammonia is detoxified by conversion to urea in the liver. The urea is then excreted in the urine. However, extrahepatic tissues do not contain a complete urea cycle. These tissues rely on the glutamine synthetase reaction to detoxify ammonia by incorporating it into glutamine (amide). In the liver, extrahepatic-derived glutamine is hydrolyzed to glutamate and ammonia, this ammonia is serving as a precursor to urea [7]. In the brain, glutamine synthetase is located in astrocytes [8,9]. During hyperammonemia, astrocyte function and morphology are compromised and astrocytes swell. Excess glutamine is now generally accepted to be a major contributing factor to this pathological process. However, the mechanism is a matter of debate [4,10–12].

A possible explanation is that excess glutamine is metabolized to a neurotoxin [13,14]. One route for the metabolism of glutamine involves its transamination to α-ketoglutaramate (KGM) followed by hydrolysis of KGM to α-ketoglutarate (KTG) and ammonia in a reaction catalyzed by ω-amidase (AMD) (the glutaminase II pathway, Scheme 1). The glutaminase II pathway is present in human brain [15]. Duffy et al. showed that there is a good correlation between the concentration of cerebrospinal fluid (CSF) KGM and degree of hepatic encephalopathy (HE) in patients with liver disease [13,14]. In fact, the correlation between KGM and degree of HE was much better for KGM than for either ammonia or glutamine. The authors noted some neurotoxicity of CSF-administered KGM in rats [13]. However, the amount of KGM administered was relatively enormous. Thus, the possibility that excess KGM is neurotoxic remains to be determined. Nevertheless,

* Corresponding author. Tel.: +1 914 594 3330; fax: +1 914 594 4058.
** Corresponding author. Tel.: +1 315 268 4421; fax: +1 315 268 6610.
E-mail addresses: arthur_cooper@nymc.edu (A.J.L. Cooper), ekatz@clarkson.edu (E. Katz).

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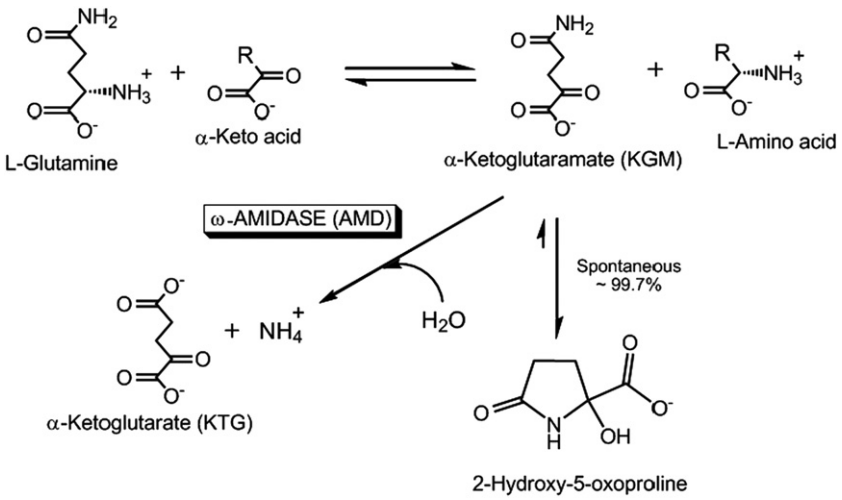


Scheme 1. The glutaminase II pathway. In the first step of the pathway, glutamine is transaminated with a suitable α-keto acid to yield α-ketoglutaramate (KGM) and an amino acid. In the second step, KGM is hydrolyzed to α-ketoglutarate (KTG) and ammonia by ω-amidase (AMD). KGM exists predominantly as a lactam. However, the reaction is reversible so that the reaction is drawn in the direction of KTG formation. During hyperammonemia, however, the AMD reaction appears to be inhibited .

the findings suggest that excess KGM may be the result of a disturbance in brain nitrogen metabolism and that KGM may be a useful biomarker for hepatic encephalopathy and other hyperammonemic diseases. However, the findings of Duffy et al. [13,14] have not been systematically followed up since the initial discovery almost 40 years ago.

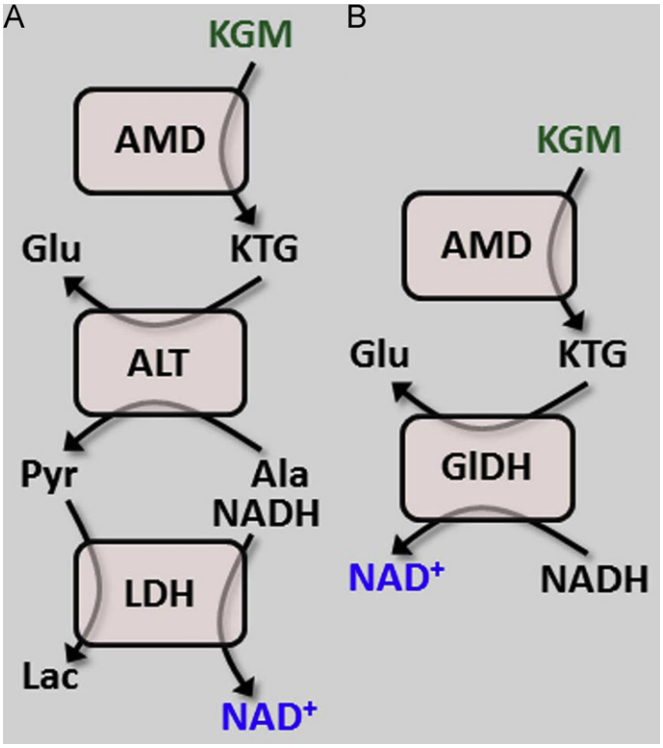
Recently, however, Kuhara et al. used a GC–MS procedure to show that KGM is markedly elevated in urine obtained from patients with primary hyperammonemia due to an inherited metabolic defect in any one of the five enzymes of the urea cycle [16]. Increased urinary KGM was also noted in other patients with primary hyperammonemia (but not in secondary hyperammonemia resulting from propionic acidemia or methylmalonic acidemia), including three patients with a defect resulting in lysinuric protein intolerance and one of two patients with a defect in the ornithine transporter I [16]. These findings together with the earlier findings of Duffy et al. [13,14] suggest that high KGM levels may be a useful surrogate for hyperammonemia in patients with HE and in patients with primary hyperammonemia due to various inborn errors of metabolism. As a first step to designing a clinically useful probe we have devised two enzymatic procedures for the measurement of KGM. The results are reported here.

2. Experimental section

Chemicals and reagents used: Alanine transaminase from porcine heart (ALT, E.C. 2.6.1.2), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), L-glutamic dehydrogenase from bovine liver (type I ammonium sulfate suspension) (GlDH, E.C. 1.4.1.2), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), α-ketoglutarate sodium salt (KTG), serum from a human male (type AB), and other standard organic/inorganic chemicals were purchased from Sigma-Aldrich and used as supplied without any further purification. Ultrapure water (18.2 MΩ cm) from a NANOpure Diamond (Barnstead) source was used in all of the experiments. ω-Amidase (AMD, E.C. 3.5.1.4) (11 U/mL; 1 mg protein/mL in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% v/v glycerol) was purified from rat liver by the method of Hersh [17] as modified by Krasnikov et al. [18] except that the last purification step was omitted. The enzyme was stored at 4 °C. When stored in 20% glycerol–phosphate buffer the purified enzyme is stable at 4 °C for at least five years. α-Ketoglutamarate (KGM) is not available commercially and no organic chemical procedure has yet been devised for its synthesis. Thus, at present the only method for its synthesis is by the method of Meister [19], first published about 60 years ago, in which L-glutamine is oxidized to KGM by snake venom L-amino acid oxidase in the presence of catalase. The free acid produced by this method is extremely deliquescent and unstable. Therefore, Meister isolated KGM from the reaction mixture as the barium salt. To avoid the necessity of removing Ba^{2+} in our studies we have modified the Meister procedure to generate KGM in situ followed by removal of protein, ammonia and unreacted L-glutamine, and addition of concentrated NaOH to a final pH of 6.0 [18]. Stock solutions of KGM were standardized by the method of Duffy et al. in which KGM is converted to α-ketoglutarate in the presence of excess highly purified rat liver ω-amidase, followed by quantitation of α-ketoglutarate by measuring the disappearance of NADH in the presence of glutamate dehydrogenase and ammonia [14]. This procedure provides the basis herein for the determination of KGM as outlined below. The KGM solution was stored at −20 °C. Solutions of KGM (sodium salt, 50–100 mM) are stable at −20 °C for at least six months. A unit of enzyme activity (U) is that amount of enzyme that catalyzes the formation of 1 μmol of product per min at 37 °C under standard assay conditions.

Instrumentation and Measurements: A Shimadzu UV-2450 UV–vis spectrophotometer, with a TCC-240 A temperature controlled holder and 1 mL poly(methyl methacrylate) (PMMA) cuvettes, was used for all optical measurements. All measurements were performed at 37.0 ± 0.2 °C, thereby mimicking the physiological temperature, and all samples were incubated at this temperature prior to measurements.

Composition of the systems: Scheme 2 shows the composition and operation of the two systems. Both systems were constructed in aqueous buffer/human serum solution mixtures which were also used as reference background solutions for all optical measurements. The background solutions were prepared from 50 mM carbonate buffer (pH 10.0) and human serum mixed at 1:1 (v/v) ratio. Note that the final pH of the mixed solution was 9.3 at 37 °C. The open-chain form of KGM (AMD substrate) exists in equilibrium with a lactam (2-hydroxy-5-oxoproline) that is not an AMD substrate. At equilibrium ~99.7% of KGM exists as a lactam and only ~0.3% exists in the open-chain configuration [17]. The interconversion between lactam and open-chain form is catalyzed by base (OH^-). At pH values ≥8.5 the rate of interconversion is very rapid and not rate determining for levels of AMD normally used in assays of the enzyme activity [17]. The first



Scheme 2. Two biocatalytic cascades for the analysis of α-ketoglutaramate (KGM). (A) A cascade based on the concerted action of ω-amidase (AMD), alanine transaminase (ALT) and lactate dehydrogenase (LDH). (B) A cascade composed of AMD, and glutamate dehydrogenase (GlDH). Note that NADH is included in the analytical system .

system investigated (AMD–ALT–LDH system; Scheme 2A) included AMD (0.66 U mL^{-1}), ALT (4 U mL^{-1}), LDH (2 U mL^{-1}), Ala (200 mM) and NADH (60 μM). The second system investigated (AMD–GlDH system; Scheme 2B) consisted of AMD (0.66 U mL^{-1}), GlDH (4.88 U mL^{-1}) and NADH (40 μM). Two additional systems were also investigated. In these systems, AMD was omitted and KTG was added to the serum/ALT–LDH or serum/GlDH reaction mixture at concentrations estimated to be generated in serum as a result of AMD activity on endogenous KGM. This simulation experiment was based on KGM concentrations estimated to be present in control serum and in serum from hyperammonemic patients. The output signal was quantified by detecting the decrease in the concentration of NADH, measured optically at $\lambda = 340$ nm ($\varepsilon_{340\ nm}$ 6.22×10^3 M^{-1} cm^{-1}) [20]. The signal is defined as the absolute value of the absorbance change.

3. Results and discussion

The goal of the present study was to optimize the AMD–ALT–LDH and AMD–GlDH enzymatic systems for the measurement of KGM in serum. Currently, there are no known endogenous inhibitors of AMD. However, we cannot rule out the possibility that inhibitors of AMD exist in biological fluids. Therefore it is important to construct standard analytical procedures for KGM in biological samples spiked with KGM of known concentrations. Consequently, in the first set of experiments two assays were performed in which known concentrations of KTG and KGM were added to human serum diluted to 50% to establish the relationship between the measured output signal and KTG and KGM concentrations (Fig. 1). The first assay was carried out with various concentrations of KTG in the presence of ALT and LDH. The second assay was carried out with various concentrations of

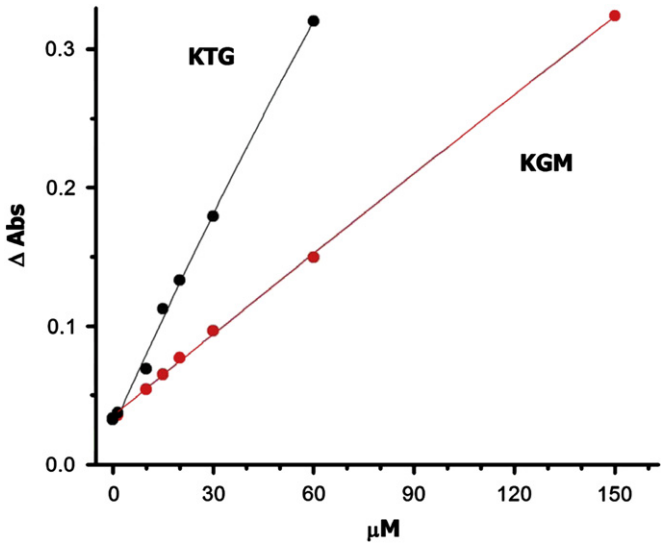


Fig. 1. Calibration curves for KTG and KGM in 50% normal human serum analyzed using the LDH–ALT system. Note that at zero concentrations of added KGM and KTG the absorbance change is not zero due to the presence of endogenous KTG in the serum.

KGM in the presence of ALT, LDH and 0.66 U mL^{-1} of AMD (to hydrolyze KGM to KTG and ammonia). The two calibration curves obtained (Fig. 1) enabled us to calculate the system response expressed as an absorbance change for any given KGM concentration from the KGM calibration curve. In a second set of experiments, two calibration curves of similar magnitude were also obtained when the GlDH system was utilized in place of the ALT–LDH system (data not shown).

Note that the slope of the KGM curve is smaller than that obtained with KTG (Fig. 1). The difference is presumably a result of incomplete conversion of KGM to KTG due to limiting AMD (Fig. S1). Nevertheless, a comparison of the KTG calibration curve versus the KGM calibration curve allowed us to calculate "true" KGM values from outputs (absorbance changes). Finally, in the next set of experiments KTG was used in the ALT–LDH and GlDH systems instead of KGM (Fig. 2). The same calibration curves were used to calculate the true KGM value based on the output from the AMD–ALT–LDH system relative to the ALT–LDH system (compare the two scales at the bottom of Fig. 2). The concentrations of KTG used in the experiment depicted in Fig. 2A and B are a proxy for values of KGM derived from the calibration curves. The "corrected" concentrations of KGM shown at the bottom of the figure correspond to expected (see below) normal physiological and pathophysiological concentrations of KGM.

To validate the system, a knowledge of expected KGM and KTG concentrations in normal and hyperammonemic human serum is required. The concetration of KTG in normal human plasma has been reported to be 23 ± 4 μM, but it is higher in hyperammonemic patients with a defect in the urea cycle [21]. Other reports suggest a plasma KTG concentration in children of 8.6 ± 2.6 μM [22]. Due to the presence of endogenous KTG in the 50% serum used as the medium in the present work, we included in the calibration curves measurement of an absorbance change when no KTG or KGM was added to the medium. Glutamate dehydrogenase is not totally specific for KTG, exhibiting low activity with other α-keto acids [23]. Thus, the control in which KGM was omitted takes into account the presence of endogenous KTG, the possible presence of substrates other than KTG in the serum, and the possibility that aminotransferases and glutamate dehydrogenase present in the serum will catalyze the conversion of NADH to NAD^+ in the presence of endogenous KTG and other α-keto

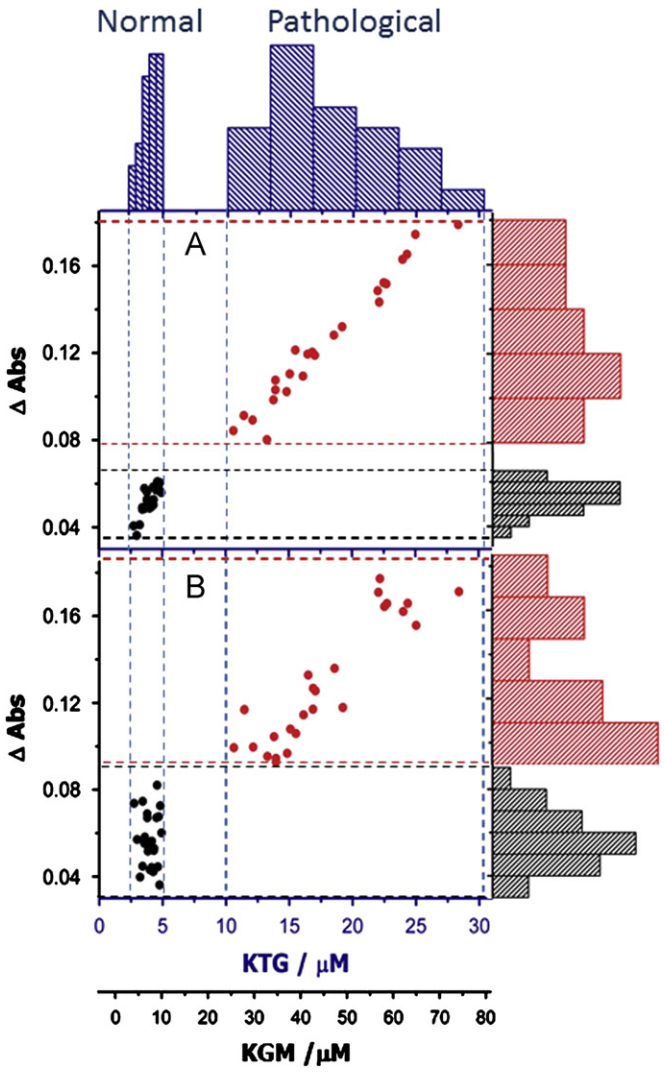


Fig. 2. Relationship between output signals measured at 600 sec and KTG concentrations, obtained for both the LDH–ALT system (A) and the GlDH system (B). The histogram juxtaposed to the top of the *x*-axis on the upper graph shows the frequency distributions of KTG concentrations used as the inputs for the control and pathological groups. Composite data from both systems were used in the construction of the histogram. The histogram juxtaposed to the right-hand *y*-axis of both plots shows the distributions of output signals. Both systems show no overlap between the output signals of the control and pathological groups. The data obtained with KTG added to the control plasma can be used as a proxy to estimate KGM concentrations expected in humans serum (horizontal scale at the bottom of the figure).

acids. Interestingly, the change in absorbance with no added KTG or KGM (~0.035; Fig. 1) suggests a maximal endogenous level of KTG in the serum of about 10–12 μM, a value consistent with plasma values reported previously [21,22]. However, no comparable data are available for plasma/serum KGM. Previous work showed that KGM is present in rat tissues at a concentration of ~5–10 μM [24]. Values for human cerebrospinal fluid (CSF) KGM were reported to be: controls (< 1–8.2 μM), liver disease patients without HE (< 1–6.2 μM) and hyperammonemic HE patients (31–115 μM) [13,14]. The relationship between human CSF KGM and serum/plasma KGM is not known. However, the ratio between CSF KTG and plasma KTG is ~0.6 [22]. Assuming a similar ratio for KGM, we assume that the concentration of KGM in human serum under normal and hyperammonemic conditions is in the range of 5–150 μM, and this range was used in construction of a calibration curve (Fig. 1).

Since the KGM concentration in human plasma/serum is not known we used as a guide published KGM concentrations established in human CSF from a control group and from patients with unequivocal symptoms of HE [13]. On inspection of the individual data points reported by Duffy et al. [13] for levels of KGM in CSF from patients with overt HE ($M=33.7\pm3.4$ μM, $n=15$ and $M=76.7\pm11.5$ μM, $n=6$) we found that the range of concentrations fitted a log-normal distribution better than a normal distribution pattern. On the other hand, the control values fitted a normal distribution. Accordingly, in order to establish a reference range for pathological serum KGM concentrations in our simulation study, we used a log-normal operation for estimated pathological values ($M_{log}=3.8\pm0.4$ μM, $n=21$) and a normal operation for estimated control values ($M=6\pm1.7$ μM, $n=16$). Using the standard R-project software R 2.1, we generated random values for normal distribution of the control group and log-normal distribution of the pathological group based on the data reported by Duffy et al. [13]. We employed 25 numbers (KGM concentrations) for each group. Using the two calibration curves (see above) for KTG and KGM in the LDH–ALT based system we calculated the values (KTG concetrations) corresponding to control and pathological serum KGM concentrations for simulated control and pathological groups. These two data sets were then used in both the LDH–ALT- and GlDH-based systems for the analysis of samples mimicking the distribution of normal and pathological KGM concentrations (Fig. 2). Importantly, the output signals measured for normal physiological concentrations of the biomarkers do not overlap with the outputs measured for the pathophysiological range of concentrations, thus allowing for complete discrimination between the two groups. It should be noted, however, that for simplicity KTG was used instead of KGM in the analytical procedure. The concentrations of KTG corresponding to the normal and pathological ranges of the KGM concentrations were obtained from the calibration plots (Fig. 1) discussed above.

4. Conclusions

The enzyme-assay protocols described herein demonstrate excellent discrimination of KGM/KTG concentrations predicted to occur in human plasma/serum between normoammonemic and hyperammonemic conditions. The robustness of the analytical assays was demonstrated by its applicability to 50%-diluted human serum solutions. In the present preliminary report, the NADH concentration (i.e. the output signal) was measured optically. Based on our previous experience with electrochemical analysis of NADH, we anticipate future transition of the present optical methods to the electrochemical domain [25]. Application of mini-invasive electrodes [26] for the electrochemical analysis of NADH would potentially allow analysis of KGM/KTG in real time in patient body fluids. It should be noted that both assays described here (i.e. AMD–ALT–LDH and AMD–GlDH) are equally good for optical sensing of KGM. However, when the analytical procedure moves to the electrochemical detection stage with enzyme-modified electrodes, the system composed of a smaller number of components immobilized on the electrodes will be more advantageous.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.022.

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