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SPECTROPHOTOMETRIC DETERMINATION OF *L*-ASCORBIC ACID IN THE HERBAL ANTIDIABETIC MIXTURES

Savych, Alona*[1]; Marchyshyn, Svitlana[1]; Mosula, Liudmila[2]; Kryskiw, Liubomyr[2]

[1]Department of Pharmacognosy with Medical Botany, I. Horbachevsky Ternopil National Medical University, Ukraine

[2]Department of Pharmachemistry, I. Horbachevsky Ternopil National Medical University, Ukraine

*alonasavych@gmail.com

Abstract

DM is a global social problem in the field of health care, due to rapid spread of this disease and the development of serious complications such as micro- and macroangiopathies, which significantly reduce the quality and life expectancy of patients.

The aim of our study was to investigate the content of *L*-ascorbic acid in HAMs with previously studied pharmacodynamics *in vivo* and *in vitro* and to validate this method of determination.

The study of *L*-ascorbic acid content in HAMs was carried out by spectrophotometry (λmax 520 nm) with the measurement in terms of USP reference standard of *L*-ascorbic acid. The method used was validated for linearity, sensitivity, precision and was established limit of detection and limit of quantification.

According to the results of spectrophotometric determination, it was established that *L*-ascorbic acid content in HAM(3) was 0.33±0.02 %, in HAM(4) – 0.30±0.02 %, in HAM(7) – 0.21±0.01 %, in HAM(13) – 0.23±0.01 % and in HAM(19) – 0.39±0.02 %.

The method was validated and showed good linearity of USP reference standard (0.02-0.1 mg/mL). The calibration curve was expressed by the regression equation y=0.0371x–0.0081, the correlation coefficient was R^2=0.9929, LOD – 0.01 mg/mL, LOQ – 0.04 mg/mL. The method used meets the characteristics of precision and accuracy.

The highest content of *L*-ascorbic acid was 0.39±0.02 % in HAM(19). The results were confirmed by the validation of method, corresponding to the characteristics of linearity, sensitivity, precision and accuracy. The data obtained indicate the presence of a correlation between the phytochemical composition of the studied HAMs and their pharmacodynamics, which was previously established.

Keywords: *herbal antidiabetic mixture, L-ascorbic acid, vitamin C, spectrophotometry, diabetes mellitus, phytotherapy, antioxidant activity, validation*

Introduction

Diabetes mellitus (DM) is a global social problem in the field of health care, due to rapid spread of this disease and the development of serious complications such as micro- and macroangiopathies, which significantly reduce the quality and life expectancy of patients [1, 2]. According to the official information of International Diabetes Federation (2019) the number of patients is projected to increase to 642 million by 2040 [3]. An important problem of pharmacovigilance is that existing pharmacotherapy cans to reduce hyperglycemia, but it is not always able to stabilize fluctuations of glycemia values during the day and keep it at optimal level [4, 5, 6]. This leads to the formation of a cascade of pathological processes – excessive glycation and inactivation of the body's antioxidant defense system, triggering free radical oxidation of lipids and, as a consequently, the development of oxidative stress, which leads to the development and progression of diabetic complications [7, 8, 9, 10]. In turn, oxidative stress is the main event for the development of insulin resistance [11, 12, 13]. This may reduce peripheral insulin sensitivity through major molecular mechanisms such as β-cell dysfunction, inflammatory responses, decreased regulation and / or localization of glucose transporter 4 (GLUT-4), mitochondrial dysfunction, and abnormal insulin signaling pathways [14, 15, 16]. Therefore, the optimization of pharmacotherapy, searching and study of new drugs with antioxidant activity for the prevention and treatment of DM and its dangerous complications is a top issue of pharmacy and medicine.

One of these areas is phytotherapy, as it has several advantages over traditional therapy, namely, it is low-toxic, has a mild pharmacological effect and possibility to be used for long periods of time without significant side effects, is well combined with synthetic drugs, has a complex activity through several biologically active compounds [17, 18, 19]. Particular attention deserves the mixtures of different medicinal plants [20, 21, 22], because such phyto-compositions are expected to have more biologically active substances that influence on different links of the pathogenetic mechanism of DM development and its complications [23, 24, 25]. Plant biocompounds have a wide range of pharmacological action and a variety influencing mechanisms on the DM and diabetic angiopathies development [26, 27, 28, 29].

In this regard, the important biologically active substance is *L*-ascorbic acid, namely vitamin C [30, 31, 32, 33]. Thus, the aim of our study was to investigate the content of *L*-ascorbic acid in some herbal antidiabetic mixtures (HAMs) with previously studied pharmacodynamics *in vivo* and *in vitro* [34, 35, 36, 37] and to validate this method.

Methods

Plant materials: The herbal raw materials harvested in June to August 2020 in Ternopil region and Charpathians (*Vaccinium myrtillus* leaf) (Ukraine) were used. After harvesting, the raw materials were dried, crushed and stored according to the general GACP requirements [38]. The plants were identified by Prof. S. M. Marchyshyn. The voucher specimens of herbal raw materials have been deposited in Herbarium of Pharmacognosy with Medical Botany Department, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine.

For the study, five different HAMs with reliable pharmacodynamics established during pharmacological studies *in vivo* and *in vitro* [34, 35, 36, 37] were used. Compositions of HAMs are given in Table 1.

Chemicals and standards: United States Pharmacopeia (USP) reference standard of *L*-ascorbic acid was of primary reference standard grade (≥ 99 % purity HPLC) and was purchased from Sigma-Aldrich Chemical Company (Germany). Oxalic acid, methanol, ethanol, thiourea, 2,6-dichloroindophenol sodium salt, 2,4-dinitrophenylhydrazine (2,4-DNPH), sulfuric acid was purchased from Sfera Sim LTD (Ukraine). Water used in the studies was produced by MilliQ Gradient water deionizaton system (USA).

Instrumentation and conditions: spectrophotometric measurements were performed on spectrophotometer UV-1800 Shimadzu (Japan) equipped with 1 cm quartz cells. Determination of *L*-ascorbic acid content was performed at a maximum absorption of 520 nm [39].

Reagents: *Reagent A* – 1.00 g of oxalic acid was dissolved in 50 mL of methanol. *Reagent B* – 100.0 g of thiourea was dissolved in 1 L of ethanol 50% (v/v). *Reagent C* – 1.5 g of 2,4-DNPH was dissolved in 50 mL of 20 % solution of sulfuric acid. *Indicator* – 50.0 mg of 2,6-dichloroindophenol sodium salt was dissolved in 100 mL of distilled water.

Test solution: 1.00 g (accurately weighed) of each powdered HAMs was placed into a round-bottomed flask. Then was added *reagent A* and heated on a water bath under reflux for 10 minutes. After that, it was cooled in an ice bath to a temperature of (15–20) ° C and filtered through a paper filter. Then 2 mL of the filtrate was transferred into a 50 mL conical flask, added sequentially, gently shaking after each addition of 2 mL of an *indicator*. Subsequently, exactly 60 s later, 0.5 mL of *reagent B* and 0.7 mL of *reagent C* were added, heated under reflux at 75 ° C and immediately placed in an ice bath for 5 minutes. Then 5 mL of a mixture consisting of 12 mL of water and 50 mL of concentrated sulfuric acid was added dropwise to the obtained solutions, adding for a period of not less than 90 s and not more than 120 s, vigorously shaking the flask in an ice bath. Maintained for 30 min at room temperature and measured the optical density using *reference solution A* as a compensating solution.

Reference solution A: 2 mL of the filtrate obtained in the preparation of the test solution was treated as described above by adding *reagent C* immediately before measuring the optics.

Standard solution: 40.0 mg (accurately weighed) of USP of *L*-ascorbic acid was dissolved in 100 mL of *reagent A*. Then 5 mL of obtained solution was placed into 100 mL volumetric flask and made up to the mark by *reagent A*. 2 mL of the resulting solution was worked up as described above for the filtrate obtained in the preparation of the test solution. The optical density was measured using *reference solution B* as a compensating solution.

Reference solution B: 2 mL of the standard solution was treated as described above by adding *reagent C* immediately before measuring the optics.

Method validation: the method was validated for linearity, sensitivity and precision (intra-assay and intermediate precision), accuracy, LOD, and LOQ [40].

Calibration curve and linearity: standard solution of *L*-ascorbic acid (2 mg/mL) was prepared in two replicate and diluted to concentrations of 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL and 0.1 mg/L. The calibration curve was constructed by plotting the measurements of mean absorbance versus the concentration of the standard solutions. The results were analyzed by linear regression and the correlation coefficient (R^2).

Sensitivity: the values for limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the data obtained during linearity testing in the low concentration range of the working in the test solution, using the following formulas: LOD = 3.3 * s / Slope; LOQ = 10 * s / Slope.

Precision and accuracy: precision was determined through the determination of intraassay and intermediate precision (40). Intra-assay precision was determined by measuring three different concentrations of each standard (prepared as two replicates, each measured three times) in one laboratory by one person. Intermediate precision was carried out by measuring the same concentrations of standards (prepared as two replicates, each measured three times) by two people in the same laboratory. Precision was expressed as percentage coefficient of variation (CV), using the following formulas: CV = standard deviation / mean x 100. Accuracy is expressed through recovery, which was determined by measuring known concentrations of standard (in three concentration levels, in triplicate).

Statistical Analysis MS Excel (Microsoft, Redmond, WA) was used for data analysis. The results for the calibration curves were based on two replicate samples of each standard concentration, each measured twice (n = 4). Regression was used to obtain the linear equation and determine R^2. Precision and recovery studies were based on the measurement of three different concentration levels, each as two replicates and each measured three times (n = 6). Ascorbic acid content in HANs was the results of two replicate samples, each measured twice (n = 4) and were presented as mean value ± SD.

Results

The results of spectrophotometric determination

of *L*-ascorbic acid content are presented in Figs. 2-3.

The method was based on conversion of *L*-ascorbic acid into coloured complex with 2,4-DNPH. The maximum absorption of all samples was at λmax = 520±0.5 nm [39].

According to spectrophotometric determination, it was established that *L*-ascorbic acid content was 0.33±0.02 % in HAM(3), 0.30±0.02 % in HAM(4), 0.21±0.01 % in HAM(7), 0.23±0.01 % in HAM(13) and 0.39±0.02 % in HAM(19) (Fig. 3).

The analytical procedure has been validated to confirm its reliability. Calibration solutions were prepared in triplicate and analyzed under the optimal conditions as described above. The calibration curve was found to be linear in the range 0.02-0.1 mg/mL for USP reference standard of *L*-ascorbic acid (Fig. 4). The regression equation ($y=0.0371x–0.0081$) and $R^2=0.9929$ revealed a good linearity response for spectrophotometric method developed for the determination of *L*-ascorbic acid content.

The results of validation showed that the LOD and the LOQ of USP reference standard of *L*-ascorbic acid was 0.01 mg/mL and 0.04 mg/mL, respectively, indicating that the sensitivity of the method was satisfactory (Table 2).

In the table 3 represents the precision and accuracy of spectrophotometric method for the determination of *L*-ascorbic acid content. CVs for the method were between 1.7 % and 3.2 % and between 1.8 and 4.5 % for intra-assay and intermediate precision, respectively. Precision was studied through intra-assay and intermediate precision. Accuracy was expressed through the determination of recovery, which was between 97.2 % and 105.1 %.

Discussion

The incidence of DM type 2 is increasing worldwide, and patients with diabetes have a greatly increased risk of death from cardiovascular disease [41, 42, 43, 44, 45]. This risk may be associated with hyperglycemia, insulin resistance, dyslipidemia, hypertension [46, 47, 48, 49]. In addition, an important pathogenetic significance in the development of cardiovascular complications in patients with DM type 2 is an increase in arterial stiffness [50, 51, 52]. Literary data indicate that *L*-ascorbic acid has ability to improve vascular elasticity, which in turn will restore microcirculation [53, 54, 55, 56].

Ascorbic acid, an antioxidant vitamin, plays an important role in protecting free radical-induced damage. Antioxidant activity of vitamin C in the treatment and prevention of DM type 2 and its complications is important because they can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation [39, 57]; scavenging ROS; inhibition the enzymes involved in ROS generation – microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, nicotinamide adenine dinucleotide phosphate (NADH) oxidase, and so forth [7, 58].

Literature sources indicate that DM type 2 reduces the level of basal vitamin C, which structurally like glucose and can replace it in many chemical reactions and thus, is effective for the prevention of nonenzymatic glycosylation of protein [30, 34, 52].

Therefore, the high content of *L*-ascorbic acid in the studied HAMs (Fig. 3) can indicate the ability of these mixtures to prevent the oxidative stress development, which is the main pathogenic mechanism of diabetic angiopathies, and can prevent the cardiovascular disease development [13, 44]. In addition, the obtained results are reliable because the method to determine *L*-ascorbic acid content in the studied HAMs was validated for linearity, sensitivity, precision (intra-assay and intermediate precision), accuracy, LOD, and LOQ (Fig. 4, Table 2-3).

The obtained data testify to the expediency of using the studied HAMs for the purpose of optimize antidiabetic pharmacotherapy.

Conclusions

The results of spectrophotometric analysis indicate the high content of *L*-ascorbic acid in the studied HAMs that provides the powerful antioxidant activity, which is an important factor in the treatment of DM type 2 and in preventing the diabetic angiopathies development. The best result of *L*-ascorbic acid content (0.39±0.02 %) was in HAM(19), which contains *Urtica dioica* L. leaf,

Taraxacum officinale L. roots, *Vaccinium myrtillus* L. leaf, *Rosa majalis* L. fruits, *Mentha piperita* L. herb.

The method to determine *L*-ascorbic acid content was validated and showed good linearity of the standard solution of USP reference standard of *L*-ascorbic acid in the range of 0.02-0.1 mg/mL, high sensitivity of the method, precision (intra-assay and intermediate precision) and accuracy.

The obtained phytochemical studies may indicate a correlation between the component composition and content of *L*-ascorbic acid in the samples of HAMs and their effectiveness in the treatment and prevention of DM type 2 and its complications.

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Table 1. Compositions of the herbal mixtures

Samples	Herbal drug component	Portion in the mixture, %	Relative ratio
HAM(3)	*Urtica dioica* leaf	26.32	5
	Cichorium intybus roots	26.32	5
	Rosa majalis fruits	21.05	4
	Elymus repens rhizome	15.79	3
	Taraxacum officinale roots	10.52	2
HAM(4)	*Arctium lappa* roots	26.32	5
	Elymus repens rhizome	26.32	5
	Zea mays columns with stigmas	21.05	4
	Helichrysum arenarium flowers	15.79	3
	Rosa majalis fruits	10.52	2
HAM(7)	*Inula helenium* rhizome with roots	10.0	1
	Helichrysi arenarium flowers	20.0	2
	Zea mays columns with stigmas	20.0	2
	Origanum vulgari herb	20.0	2
	Rosa majalis fruits	20.0	2
	Taraxacum officinale roots	10.0	1
HAM(13)	*Cichorium intybus* roots	26.32	5
	Elymus repens rhizome	26.32	5
	Helichrysum arenarium flowers	21.05	4
	Rosa majalis fruits	15.79	3
	Zea mays columns with stigmas	10.52	2
HAM(19)	*Urtica dioica* leaf	20.0	1
	Taraxacum officinale roots	20.0	1
	Vaccinium myrtillus leaf	20.0	1
	Rosa majalis fruits	20.0	1
	Mentha piperita herb	20.0	1

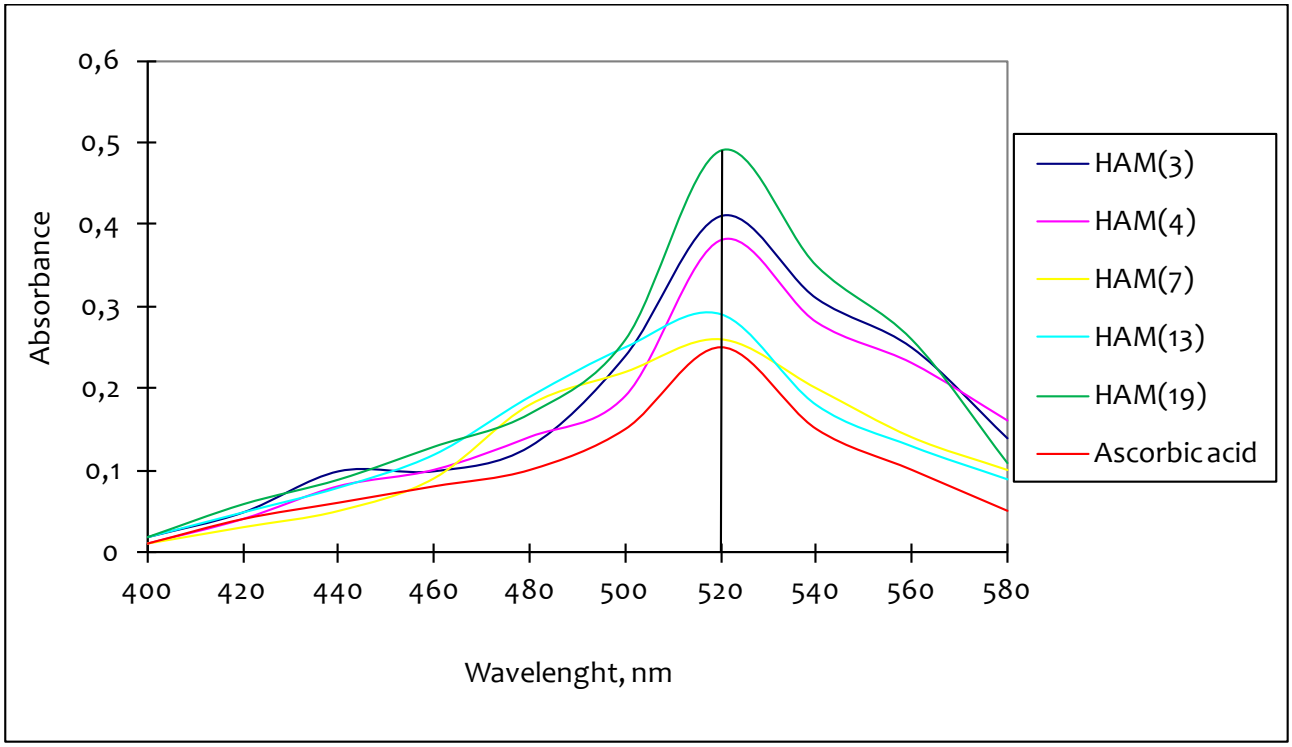

Figure 1. *Absorption spectra of L-ascorbic acid content in HAMs. Values are expressed as mean ± SD (n=4).*

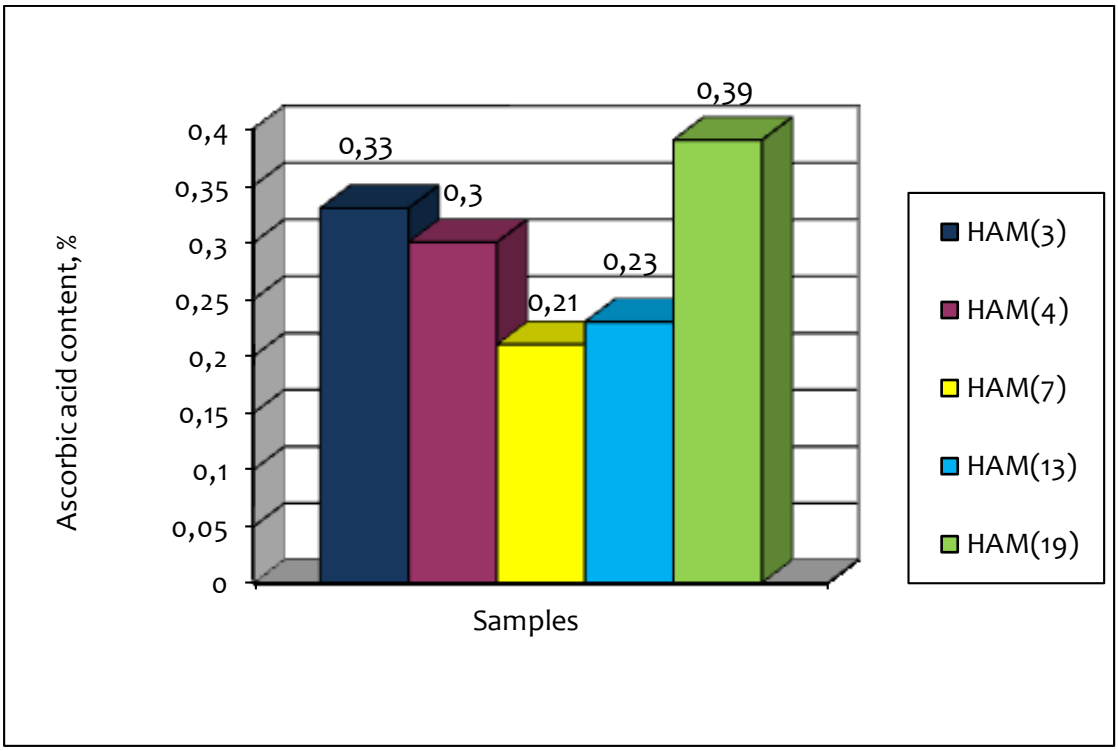

Figure 2. Ascorbic acid content in HAMs by spectrophotometric assay. Values are expressed as mean ± SD (n=4).

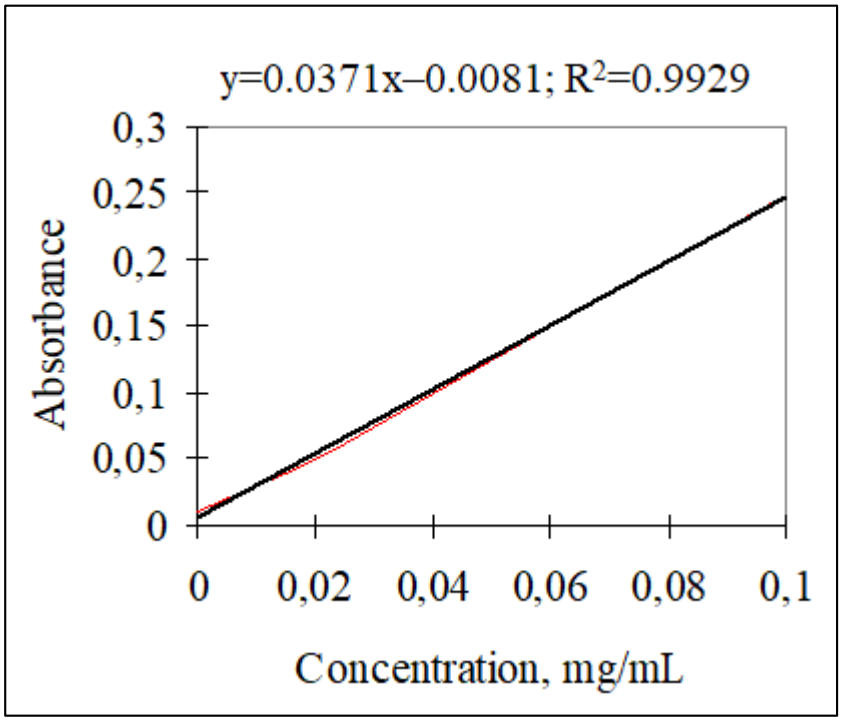

Figure 3. Calibration curve of standard solution of *L*-ascorbic acid.

Table 2. Linearity, LOD and LOQ of the spectrophotometric method for the determination of *L*-ascorbic acid content

Standard	Range, mg/mL	Regression curve	R^2	LOD, mg/mL	LOQ, mg/Ml
L-ascorbic acid	0.02-0.1	y=0.0371x–0.0081	0.9929	0.01	0.04

Note: Results are based on two replicate samples of each standard concentration, each measured twice (n = 4).

Table 3. Precision and accuracy of the spectrophotometric method for the determination of *L*-ascorbic acid content

Standard	Concetration, mg/mL	Intra-assay precision*, %	Intermediate precision**, %	Recovery, %
L-ascorbic acid	0.25	1.7	1.8	97.2
	0.50	3.2	4.5	104.2
	1.00	2.4	2.9	105.1

Note:

***** intra-assay precision – one analyst in one laboratory, with each concentration made as two replicates and each measured three times (n = 6);

****** intermediate precision – two analysts on different days in the same laboratory, with each concentration made as two replicates and each measured three times (n = 6).