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A rapid method for detection of Aldehyde-based flavour compounds in *Polygonum minus* cultured tissue

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Abstract. A rapid method for qualitative determination of aldehyde compounds was developed based on the Tollens' test. The test was carried out by first mixing a micro quantity (100 µl) of standard solutions in pentane and Tollens' reagent (10% $AgNO_3$: 10%NaOH: 2%NH_3 solution, 1:1: drops) or ammoniacal silver nitrate in wells of a microtitre plate. Then, the plate was incubated in an environmental incubator-shaker for 15 min at 45°C while shaking at 300 rpm. The plate was further incubated in a fan-ventilated oven at 70°C for another 15 min. After the incubation period a resultant brown solution was assessed visually or by using a multiscan spectrophotometer at 492 nm. The micro quantity of reagents was used so that the reaction will only proceed to a browning reaction instead of a silver mirror formation. The modified Tollens' test was found to be able to detect aldehyde compounds at a minimum level of 500 ppm. The method developed was applied to detect the presence of aldehyde flavour in *Polygonum minus* leaves and callus. The presence of aldehyde compounds in test samples was further confirmed by a gas chromatography (GC) technique. Generally, comparative results were observed whereby positive samples containing aldehyde flavour as detected by the modified Tollens' test are also positive as detected by GC. Thus, the technique could be useful as a screening procedure to detect the production of aldehyde flavour by various clones of cultured *Polygonum minus* tissue.

Abstrak. Suatu kaedah cepat bagi mengesan sebatian aldehid secara kualitatif telah dibangunkan berasaskan kaedah ujian Tollens. Ujian dimulakan dengan mencampurkan sejumlah kecil (100 µl) larutan piawai (undekanal) dalam pentana dan reagen Tollens (larutan 10% $AgNO_3$: 10%NaOH: 2%NH_3, 1:1:1: titisan) atau larutan argentam nitrat beramonia dalam telaga-telaga plat mikrotiter. Kemudian, plat berkenan dieram dalam alat pengeram-berpenggoncang persekitaran terkawal selama 15 min pada suhu 45°C dengan kelajuan penggoncang, 300 rpm. Plat itu selanjutnya dieram dalam ketuhar berkipas pada suhu 70°C selama 15 min. Selepas itu larutan kecoklatan yang terbentuk dinilai secara penglihatan mata kasar atau menggunakan spektrofotometer multiskan pada panjang gelombang 492 nm. Kuantiti mikro reagent perlu digunakan supaya tindak balas hanya berlangsung ke tahap pembentukan larutan perang dan bukan pembentukan cermin perak. Ujian Tollens terubahsuai ini didapati berjaya mengesan sebatian aldehid pada aras serendah 500 bpj. Kaedah yang dibangunkan itu telah digunakan untuk mengesan sebatian perisa aldehid dalam sample daun dan kalus *Polygonum minus* (kesom). Kehadiran sebatian aldehid dalam sampel yang diuji seterusnya disahkan dengan teknik kromatografi gas (GC). Umumnya, keputusan yang setanding telah dicerap yang mana sampel yang positive mengandungi perisa aldehid menerusi ujian Tollens terubahsuai juga positive bila dikesan dengan GC. Maka, kaedah ujian yang dibangunkan ini amat berguna untuk tujuan penabiran kehadiran sebatian perisa aldehid dalam klon tisu kultur *Polygonum minus.*

Key words: Tollens' test, GC, aldehyde, *Polygonum minus* callus

Introduction

Preliminary detection of aldehyde flavour compounds produced by cultured plant tissues or calli is essential before proceed to the actual detection using a more objective technique, the GC technique. This is to ensure that all calli chosen from hundreds of clones for further treatment are significantly producing the compounds. Several chemical methods for detecting the aldehyde can be employed such as the reducing reactions using 2,4 dinitrophenylhydrazine or triiomethane reagents, or the oxidation reactions using Fehling or Tollens' reagents [1]. Tollens' test is a chemical reactions which is a reduction of Tollens' reagent, ammoniacal silver nitrate, by an aldehyde group producing silver mirror on the inside wall of a glass reaction vessel. However, the formation of silver mirror is very much determined by several factors include temperature, pH, concentration of substrate (aldehydes or ketones), water activity (a_w), moisture and time [2].

Trace amount of substrate, generally denies the formation of silver mirror in the Tollens' test. Instead, the reaction under certain conditions may equivalent to the browning Millard reaction. This is true as described in a model system of acetol-ammonium sulfate by Linda *et al.* [3]. In this study the original Tollens test for aldehyde and ketones is optimised so that the reaction can be used to detect trace amount of aldehyde as equivalent to the browning Millard reaction.

Materials and Method

Samples preparation

Aldehyde flavour compounds were extracted from *Polygonum minus* Huds. (kesom) leaves and cultured tissues or callus using a distillation technique in Likens-Nickerson extraction apparatus [4]. Calli were prepared from at least 5 different clones grown on different media [5]. N-pentane was used to trap any kesom flavour liberated during extraction. The pentane solution was then concentrated to ensure the detection of small amount of kesom flavour, and is ready to be analysed using a modified Tollens' test.

Preparation of Tollens' reagent

Fresh Tollens' reagent was prepared as described by Fieser and Williamson [6] with some modifications. This was carried out by gentle mixing 1 ml of 10% $AgNO_3$ solution and 1 ml of 10% NaOH solution in a thoroughly clean test tube. Consequently, suspended silver oxide will be formed. A few drops of 2% dilute ammonia (NH_3) solution was then added slowly until the silver oxide just dissolves.

Optimization of test condition

An initial experiment was carried out to determine optimum conditions (time and temperature) for the Tollens' test. An amount of 100 µl Tollens' reagent was added to each of 100 µl standard (undecanal) solutions of different concentration (0 – 5000 ppm) in wells of a microtitre plate. Concentrated undecanal used as standard was diluted in pentane. The plate was then incubated at 45°C in an environmental shaker, shaking at 300 rpm, for 15 min. Subsequently, the plate was transferred in an oven at 60, 70 or 80°C for 15, 30 or 45 min. After incubation, the reaction was stopped by cooling the plate down to room temperature or lower. The intensity of brown solution formed was measured using a multiscan spectrophotometer (Anthos Reader 2001) at 492 nm.

Sensitivity of the modified Tollens' test

The sensitivity of the modified Tollens' test was determined by reacting the reagent with a series of known concentration (0 – 2000 ppm) of standard (undecanal) solution. 100 µl of Tollens' reagent was added into wells of microtitre plate containing 100 µl of standard undecanal solution each. The plate was then incubated in an environmental shaker (300 rpm) at 45°C for 15 min. The plate was further incubated in a fan-ventilated oven at 70°C for another 15 min. After the incubation, the plate was cooled down to room temperature. Finally, the absorbance at 492nm of the reaction product in each wells of the plate was measured. A standard curve was drawn whereby the various concentrations of the aldehyde is plot against the absorbance (at 492 nm) of the reaction product. The least concentration of the standard undecanal that gives absorbance significantly different from a blank solution indicates the sensitivity of the test.

Application of the modified Tollens' test

The test was applied to all pentane extracts of kesom leaves and cultured tissues or callus. Extract of kesom flavor was prepared using steam distillation technique in a Likens-Nickerson apparatus and the flavour was trapped in pentane. The test was carried out according to the method described for standard undecanal solution above.

The ability of the modified Tollens' test to detect aldehyde-based flavour compounds in kesom tissue extracts was compared with a standard gas chromatography technique. The GC technique was standardised as follows:

GC Model : Shitmatzu Model 17A with FID system
Column : Non-polar DPX 1
Column size : 0.25 mm ID x 50 m
Gas flow rate: 1.3 ml/min

Temperature for
Detector : 280°C
Injecto r: 250°C
Oven: 70 – 200°C (at 4°C/min)
Carrier gas : Helium

Results and Discussion

Tollens' test for aldehyde

A reaction of Tollens' reagent, an alkaline solution of silver ammonium hydroxide, and an aldehyde leads to reduction of the reagent to metallic silver and oxidation of the aldehyde to a carboxylic acid [6]:

$$[H_3N - Ag - NH3]^+OH^- + R - CH = O \xrightarrow{NaOH} Ag^0 + R - COONa + 2NH_3 + H_2O$$

The reaction is also known as a silver mirror test for aldehyde compounds.

Modified Tollens' test

The modified test involved an initial reaction of Tollens' reagent and the aldehyde at 45°C for 15 min in an environmental shaker running at 300 rpm in order to initiate the formation of amino-aldehyde complexes before the formation of Schiff's bases and hence the brown pigment or colouration. Further incubation in oven was carried out in an oven at

Table 1: Detection of aldehyde using the Tollens' test at different temperatures.

Time (min) of oven incubation at 60°C,	Intensity of brown colouration of reaction product [Concentration of standard undecanal, ppm]					
	[5000]	[2000]	[1000]	[500]	[100]	[0]
15	¤¤¤¤	¤¤¤	¤¤	¤	+	Cls
30	++++	+++	++	+	+	+
40	++++	+++	++	+	+	+
incubation at 70°C						
15	¤¤¤¤	¤¤¤	¤¤	¤	¤	Cls
30	++++	+++	++	+	+	+
45	++++	+++	++	+	+	+
incubation at 80°C						
15	¤¤¤¤	¤¤	¤	¤	+	Cls
30	++++	+++	++	+	+	+
45	++++	+++	++	+	+	+

Key for colour intensity:

¤¤¤¤	Very dark brown	++++	Dark gray	Cls	Clear solution
¤¤¤	Dark brown	+++	Gray		
¤¤	Brown	++	light gray		
¤	Light brown	+	Very light gray		

Table 2: Detection of aldehyde using the modified Tollens' test and GC technique

Sample of *kesom* tissue	Modified Tollens' Test	GC Detection		
		Peak of Chromatogram	Retention time (min) of peak	Standard aldehyde equivalent
Cal-1-Try	-ve	no peak	-	-
Cal-2-Try	-ve	no peak	-	-
Cal-3-Try	-ve	no peak	-	-
Cal-4-Try	+ve	peak	30.25	undecanal
Cal-5-Try	-ve	no peak	-	-
Cal-1-Ala	+ve	peak	21.40; 31.12	decanal; undecanal
Cal-2-Ala	-ve	no peak	-	-
Cal-3-Ala	+ve	peak	21.46; 30.14	decanal; undecanal
Cal-4-Ala	+ve	peak	21.38; 30.09	decanal; undecanal
Cal-5-Ala	+ve	peak	20.43; 29.44	decanal; undecanal
Cal-1-Asp	+ve	peak	30.82	undecanal
Cal-2-Asp	+ve	peak	30.86	undecanal
Cal-3-Asp	+ve	peak	30.90	undecanal
Cal-4-Asp	-ve	no peak	-	-
Cal-5-Asp	+ve	peak	30.80	undecanal
Kesom leaf	+ve	peak	30.45	undecanal

Key:
-ve clear solution of reaction product
+ve brown solution of reaction product
GC Gas chromatography
Cal- -Try Callus grown on media containing tryptophane
Cal- -Ala Callus grown on media containing alanine
Cal- -Asp Callus grown on media containing aspartic acid

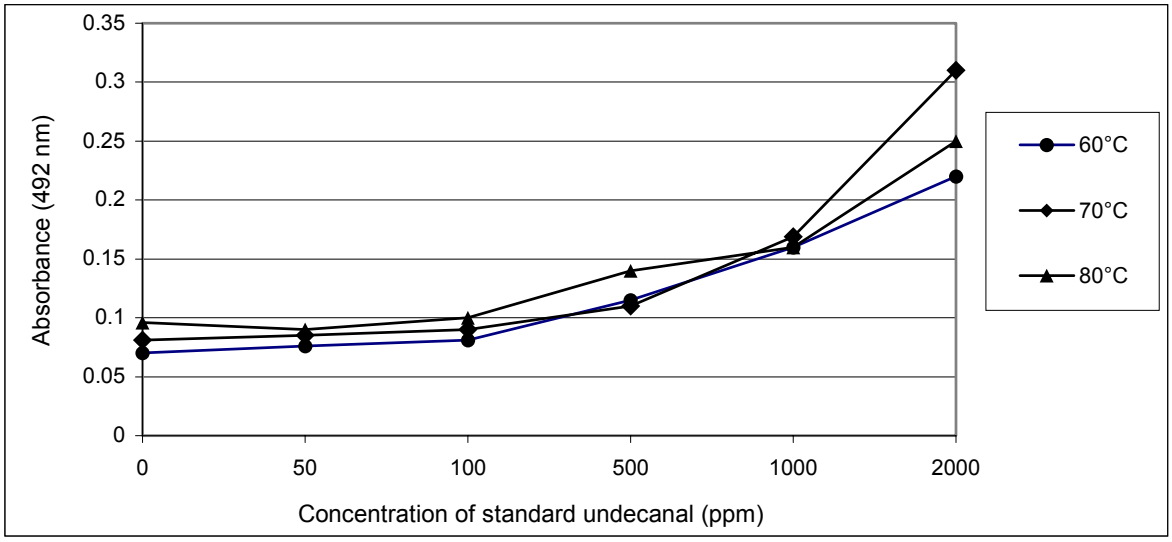

Figure 1: Detection of Standard Undecanal using the Tollens' test at different temperature

different temperature and time of incubation (Table 1). The Tollens' test for aldehydes was modified in such away that the reaction would resemble the Millard reaction. Many researchers have established that a reaction between ammonia or ammonium compounds and aldehydes such as reducing sugars is actually equivalent to the Millard or browning reaction [3, 7, 8, 9].

Visual observation indicated that an optimum condition for oven incubation is at 70°C for 15 min (Table 1). Reaction at other temperature and time did not producing a distinct brown colouration of the reaction product and blank should be a clear solution. Only grayish solution was observed. Figure 1 is plots of undecanal concentrations against absorbance at 492 nm which indicates reaction at 70°C for 15 min produce product with highest absorbance.

A series of standard undecanal concentrations (0 – 2000 ppm) was prepared and reacted with Tollens' reagent, to develop a standard curve (Figure 2) for a semi-quantitative detection of aldehyde in a sample solution. From the standard curve (Figure 2) it can be deduced that the test was sensitive to a solution containing not less than 500 ppm aldehyde. Therefore, the modified Tollens' test should be reliable when it is used to detect the presence of 500 ppm aldehyde or more in a sample solution.

Detection of aldehyde-based flavour compounds

The modified Tollens' test was applied in screening the presence of aldehyde-based kesom flavour extracted from cultured kesom tissues or callus (Cal-1, 2, 3, 4 and 5-Try; Cal-1, 2, 3, 4 and 5-Ala; Cal-1, 2, 3, 4 and 5-Asp) and leaves. Table 2 shows visual detection of the aldehyde flavour in the extracts using modified Tollens' test as compared to the more accurate GC detection. Generally, the results were reliably comparable to the GC technique. Positive test for callus or leaf extract showed the presence of the corresponding aldehyde flavour as detected by the GC technique.

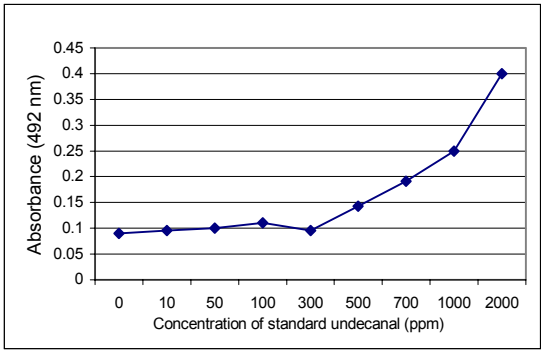

Figure 2: A standard curve for the intensity of brown colour produced when Tollens' reagent reacts with undecanal.

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