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Identification of Oxidation Products of L-Ascorbic Acid by HPLC

Linda Lloyd
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Ascorbic acid is a water soluble sugar acid with antioxidant properties. The L-isomer of ascorbic acid is commonly known as vitamin C and is found naturally in fruits and vegetables. It is also added to fruit juices and other processed products as an antioxidant. Vitamin C is an essential nutrient in the human diet in the manufacture of collagen. In humans, absence of the vitamin leads to scurvy, a deficiency disease.

The acid has strong reducing power but when oxidized is converted to several compounds that do not have the same antiscorbutic or reducing properties. Given the importance of the vitamin in human health and its widespread use as an antioxidant in processed foods, study of its degradation products is merited. This note describes aspects of the rate of degradation of L-ascorbic acid and the nature of some of its degradation products using PLRP-S columns from Polymer Laboratories, now a part of Varian, Inc. PLRP-S is a rigid macroporous styrene/divinylbenzene HPLC phase with outstanding chemical and physical stability. The high surface area of the 100Å pore size enables retention of water soluble solutes.

The aim of this study was to develop a rapid HPLC method for the quantitative and qualitative analysis of L-ascorbic acid.

Materials and Reagents

Reference samples: commercial L-ascorbic acid, oxalic acid, L-dehydroascorbic acid (DHAA) dimer

Conditions

Columns: 2 x PLRP-S 100Å 5 µm, 150 x 4.6 mm
Eluent: 0.2 M NaH_2PO_4, pH 2.14
Flow rate: 0.5 mL/min
Inj Vol: 20 µL
Detector UV, 268 and 220 nm

Results and Discussion

Reference materials

The retention of reference compounds is shown in Figure 1. L-ascorbic acid was well resolved from its possible degradation products.

Bromine oxidation of L-ascorbic acid

HPLC of freshly prepared solutions of L-ascorbic acid treated with bromine produced the curves shown in Figure 2. Peak

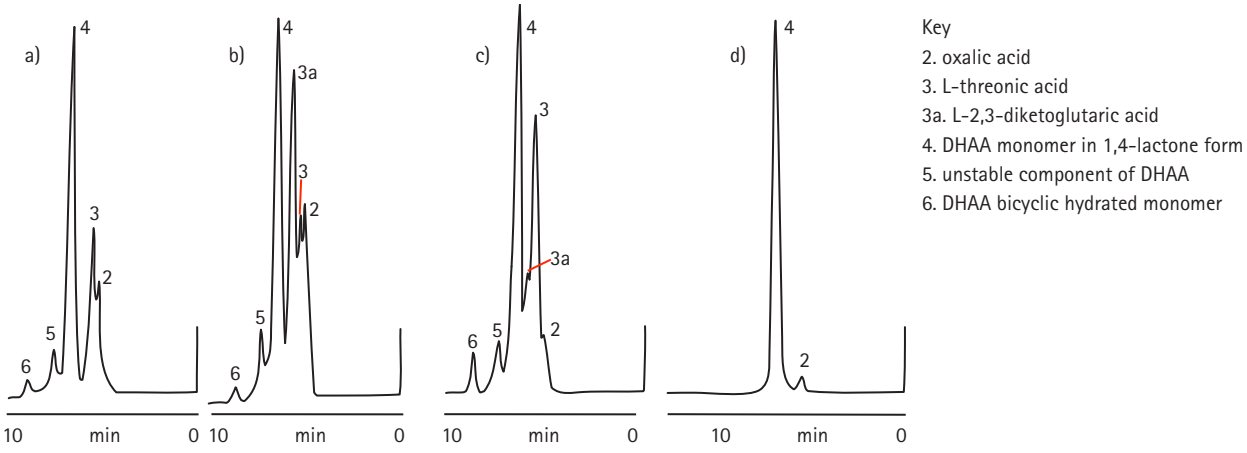

Figure 1. HPLC separation of a) L-dehydroascorbic acid solution spiked with b) potassium L-2,3-diketoglutaric acid and c) calcium threonate reference materials. d) is L-dehydroascorbic acid produced by Dietz's method, at 220 nm (0.2 AUFS).

1 is a high response at 220 nm, whereas peak 2 is a smaller response later identified as DHAA monomer with a free side chain. Peak height intensity was proportional to the amount of bromine added. Further addition of bromine was made to a cold solution of L-ascorbic acid (2 °C) to control degradation of DHAA. Homocysteine was then added and the reduction reaction went ahead at room temperature.

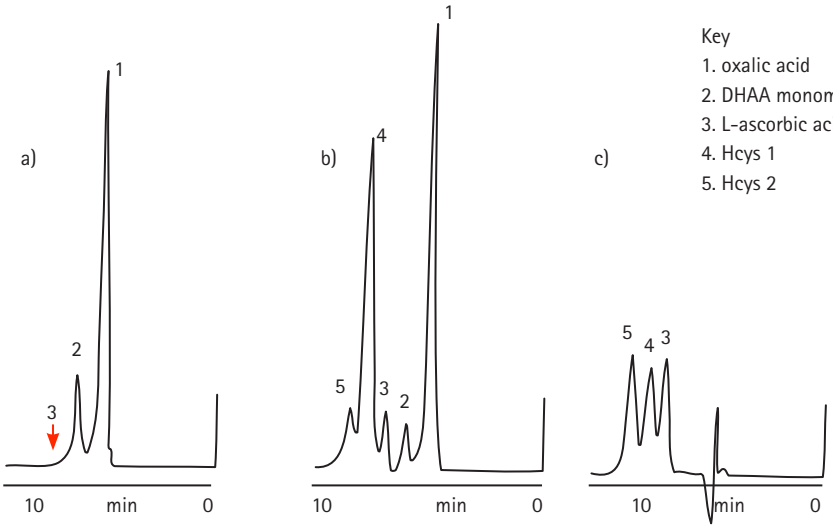

Figure 2. HPLC separation of a) bromine-oxidized L-ascorbic acid, at 220 nm (0.2 AUFS), and b) after addition of homocysteine (1:1), at 220 nm (0.1 AUFS) and c) at 268 nm (0.02 AUFS).

Effect of homocysteine on DHAA

The reaction of homocysteine with DHAA was examined to distinguish the DHAA peak from surrounding peaks in the commercial samples (Figure 3). *Inter alia*, L-ascorbic acid (peak 4a) increased very rapidly after reaction with homocysteine, and peaks 4 and 6 represent monomeric forms of DHAA.

The complete data set and analysis is available in Kennedy *et al.* (1989).

Conclusion

PLRP-S columns successfully revealed the identities of some degradation products of vitamin C in the development of a qualitative and quantitative HPLC method for the fast analysis of L-ascorbic acid.

Reference

Kennedy, JF, White, CA, Warner, FP, Lloyd, LL and Rivera, ZS (1989) The identification and analysis of the oxidation products of L-ascorbic acid by HPLC C. *J. Micronut. Anal.*, **5**, 91-109.

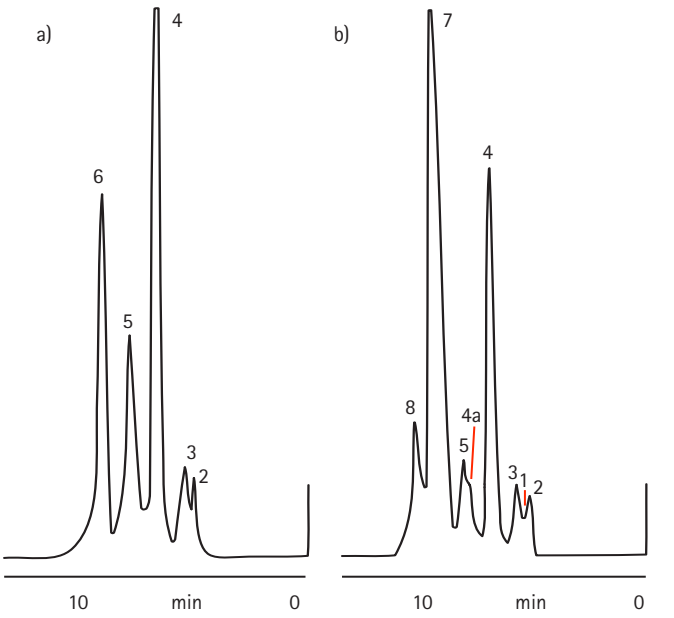

Figure 3. HPLC separation of L-dehydroascorbic acid solution a) before and b) after reduction reaction with homocysteine, at 220 nm (0.05 AUFS), using optimized conditions.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Application Note SI-01474

Accurate analysis of organic acids and alcohols by HPLC

Linda Lloyd
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Hi-Plex H columns from Polymer Laboratories, now a part of Varian, Inc., enable the separation of glucose, fructose and malic acid from wine while avoiding the common problem of the malic acid co-eluting with the glucose, which results in a false indication of sweetness. In addition, analysis of malic acid can show the age of a wine. Hi-Plex H is also the column of choice for the analysis of sugar alcohols and sugar molecules.

Instrumentation

Figure 1
Column: Hi-Plex H 8 µm, 300 x 7.7 mm
Hi-Plex H Fast Acid 8 µm, 100 x 7.7 mm
Hi-Plex H Guard, 50 x 7.7 mm
Detector: RI

Figure 2
Column: Hi-Plex H, 300 x 7.7 mm
Detector: UV, 210 nm

Materials and Reagents

Figure 1
Eluent: 0.005 M H_2SO_4

Figure 2
Eluent: 5 mM H_2SO_4

Conditions

Experimental conditions, specifically temperature, must be carefully optimized to ensure separation and prevent co-elution. The strength of the acid eluent also affects the resolution. The optimum temperature for resolution is greatly reduced by changing from 5 mM H_2SO_4 to 4 mM H_2SO_4.

Figure 1
Flow Rate: 0.6 mL/min
Temp: 76 °C

Figure 2
Flow Rate: 0.6 mL/min
Temp: 55 °C

Results and Discussion

Figure 1 shows good separation of three constituents of wine, and Figure 2 reveals the excellent separation of six organic acids, both separations using Hi-Plex H columns.

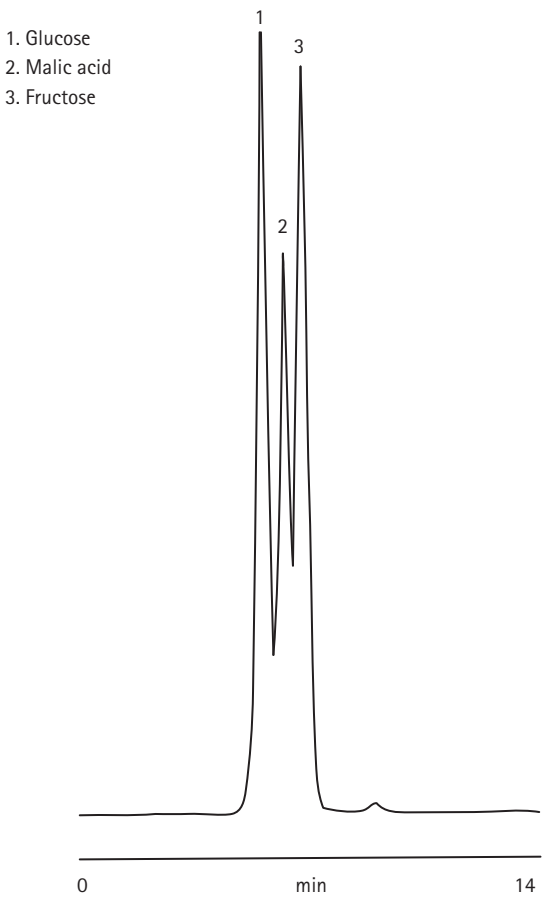

Figure 1. Good separation of wine components using PL Hi-Plex H columns, with no co-elution of malic acid and glucose.

Key

1. Oxalic Acid
2. Citric Acid
3. Malic Acid
4. Succinic Acid
5. Formic Acid
6. Acetic Acid

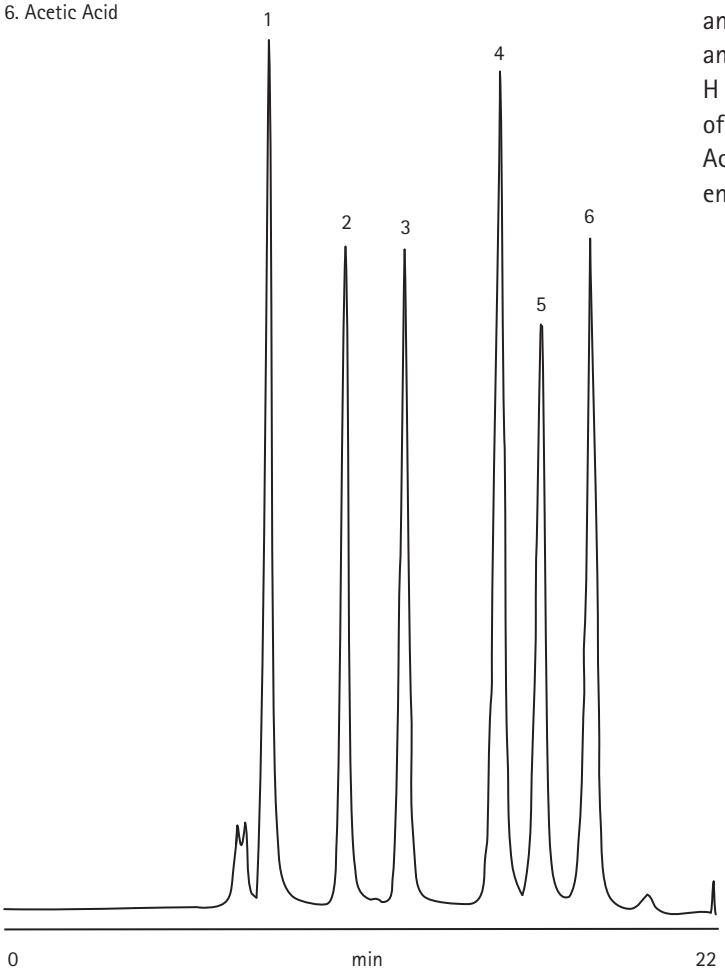

Figure 2. Hi-Plex H columns resolve organic acids with very good separation.

Conclusion

Hi-Plex columns are packed with sulfonated resin giving a fundamental improvement in performance. The monodisperse packing overcomes the problems of low efficiencies and high back pressures encountered with 'soft' gels. The columns are available in hydrogen form for the analysis of organic acids and alcohols. Hi-Plex H is also the column of choice for the analysis of sugar alcohols and sugar molecules. The Hi-Plex H Fast Acid column is also available for direct injecting of samples onto the column, with results within minutes. Accurate determination of composition and content is ensured.

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Application Note SI-01411 / TB2004/2005

Profiling juice carbohydrates with evaporative light scattering detection

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

The predominant sugars in fruit juice are the monosaccharides, glucose and fructose, and the disaccharide, sucrose. The ratio of these three sugars differs in each type of fruit juice. However, for each single fruit the ratio is relatively constant. In the production of "pure" fruit juices, the juice must be extracted by mechanical means and must not contain any added material. The mechanical extraction and concentration of the juice is normally carried out in the country where the fruit is grown. The concentrate is shipped to an intermediate country or the country where the juice is to be consumed, where it is reconstituted to produce the "pure" fruit juice product. The juice concentrate is an expensive commodity and one way of increasing profit is to extend the juice by the addition of sucrose and water, which are considerably cheaper. This extension of the juice is a form of adulteration, not detectable by the measurement of °Brix, as this assesses total dissolved sugar and not the ratio of the individual sugars.

Instrumentation

Hi-Plex resins from Polymer Laboratories, now a part of Varian, Inc., are ideal for the analysis of processed foods that contain a significant quantity of sugar, and provide a profile of both naturally occurring and added sugars. To determine the ratio of sucrose, glucose and fructose, a rapid, reliable and robust method is required. There are a number of HPLC methods to quantify these three sugars, with one of the most simple being the use of a calcium ligand exchange column, Hi-Plex Ca, with water as the eluent.

Sugars do not have a UV chromophore and therefore the RI detector has to be used if the solutes are to be analyzed without any derivatization. An improvement in sensitivity for carbohydrates can be achieved by using an evaporative light scattering detector, PL-ELS 1000, also from Polymer Laboratories. In addition to an increase in sensitivity, this detector gives a more stable, drift-free baseline which can improve the precision of the quantitation.

Column: Hi-Plex Ca, 300 x 7.7 mm
Detector: RI or PL-ELS 1000 (neb=80 °C, evap=80 °C, gas=1.0 SLM)

Materials and Reagents

Eluent: Water

Conditions

Flow Rate: 0.6mL/min
Temp: 85 °C

Results and Discussion

Figures 1 and 2 show the separation of orange juice and apple juice, respectively, and clearly indicate that the ratio of the three sugars differs in the two juices, sucrose being the main sugar in the orange juice and fructose in the apple juice. The superior stability provided by the PL-ELS 1000 ELSD is evident in the carbohydrate separations of two additional samples of orange and apple juice, as shown in Figure 3 and Figure 4.

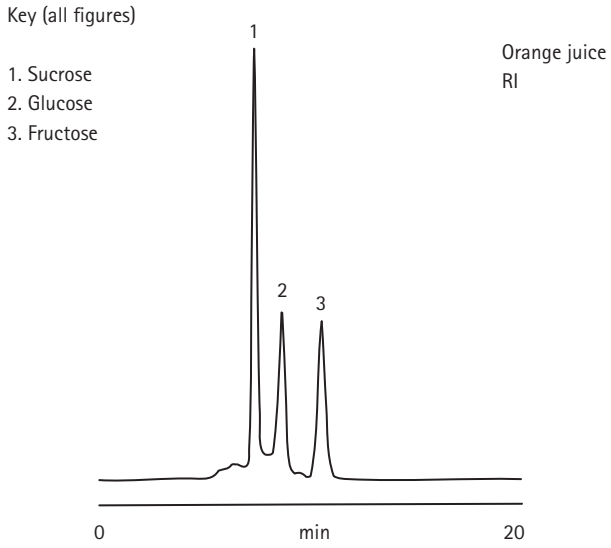

Figure 1. Sugar ratios in orange juice revealed by HPLC with a Hi-Plex Ca column and RI detection.

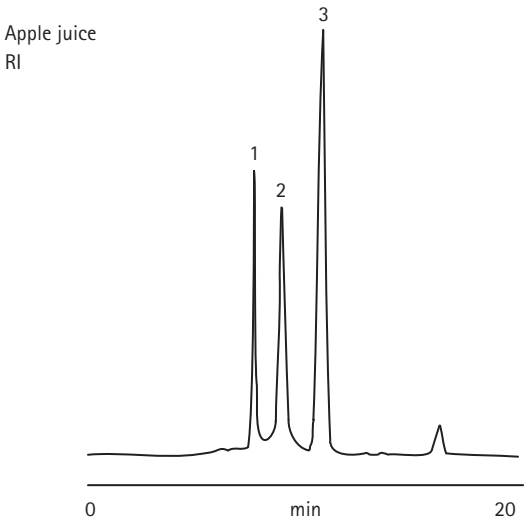

Figure 2. Sugars ratios in apple juice revealed by HPLC with a Hi-Plex Ca column and RI detection.

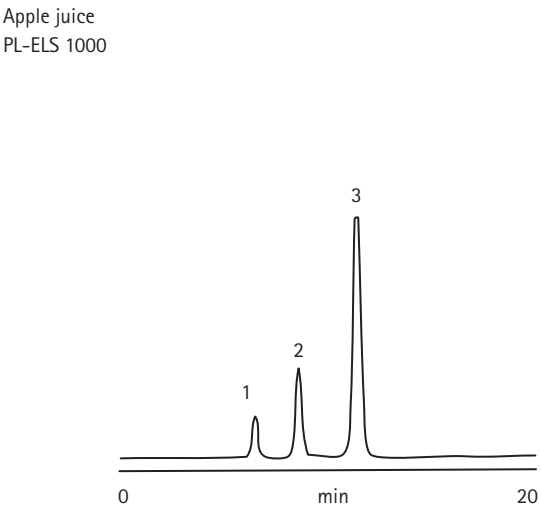

Figure 3. Sugar ratios in apple juice revealed by HPLC with a Hi-Plex Ca column and PL-ELS 1000 evaporative light scattering detector.

Conclusion

Hi-Plex columns are packed with sulfonated resin giving a fundamental improvement in performance to overcome the problems of low efficiencies and high back pressures encountered with 'soft' gels. The columns are available in calcium form for the analysis of carbohydrates in fruit juices, to meet the growing demand for more detailed product information for labelling and control purposes. The robust design of the PL-ELS 1000 evaporative light scattering detector allows the nebulizer and evaporator to operate at very high temperatures, efficiently handling the high boiling point solvents for this type of analysis that other ELSDs simply cannot manage. By using Hi-Plex columns and the PL-ELS 1000 ELSD, accurate determination of composition and content is assured.

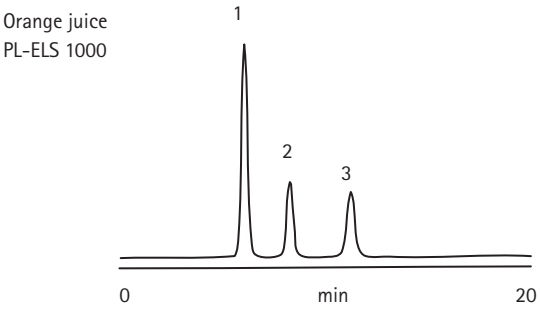

Figure 4. Sugar ratios in orange juice revealed by HPLC with a Hi-Plex Ca column and PL-ELS 1000 evaporative light scattering detector.

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Application Note SI-01408 / TB2001

Application Note SI-01151

Hi-Plex columns for fingerprinting organic acids in wine

Stephen Ball
Polymer Laboratories, now part of Varian, Inc.

Introduction

The Hi-Plex H is a high performance ligand exchange chromatography column from Polymer Laboratories, now a part of Varian, Inc. The column is based on polystyrene divinylbenzene with an 8% crosslinking and hydrogen counter ion. Typically used for the analysis of sugars, sugar alcohols and organic acids, its monodisperse sulfonated packing gives improved column efficiency, lower column pressure and assured batch to batch reproducibility.

The superior separation ability of Hi-Plex H is demonstrated in the quantitative analysis of organic acids in four different samples of wine: red, white, rosé and dessert wine. This type of analysis is important for the wine quality control, because the classes and content of organic acids give a characteristic taste to the finished product. Acetic acid, lactic acid, succinic acid, malic acid, citric acid and tartaric acid are the main organic acids in wine.

The use of a ligand exchange chromatography column such as Hi-Plex H significantly reduces the need for complicated sample preparation (typically involving elution through an ion exchange resin bed), as retention is brought about by not only ion exchange, but also by ion exclusion and partitioning on this type of column.

Materials and Reagents

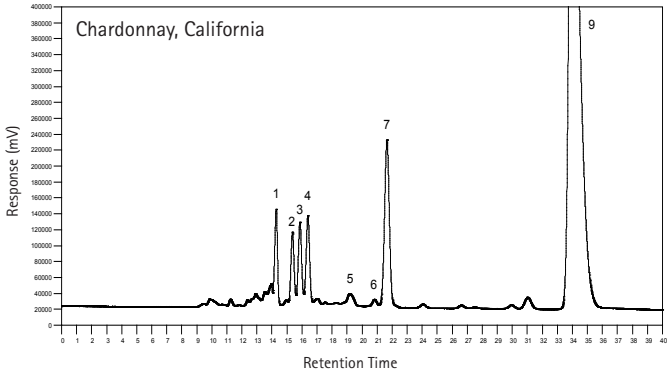
Columns: Hi-Plex H, 8 µm, 300 x 7.7 mm
(PL1170-6830)

Sample Preparation

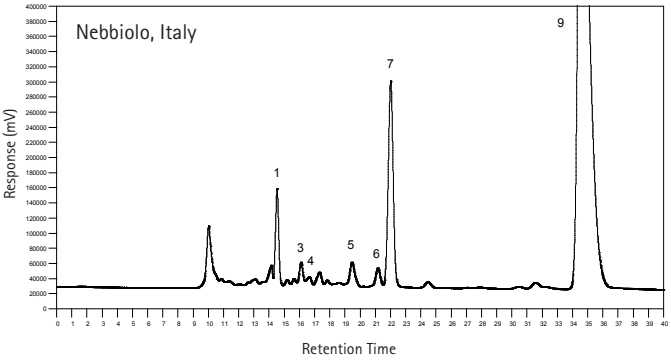
In order to obtain a complete RI profile, each wine was directly injected onto the column without any sample pre-treatment. The only exception to this being the Inniskillin Eiswein which was diluted by a factor of five with HPLC grade water, and the Marsala Wine which was diluted by a factor of three with water. Injection volume was 20 µL.

Conditions

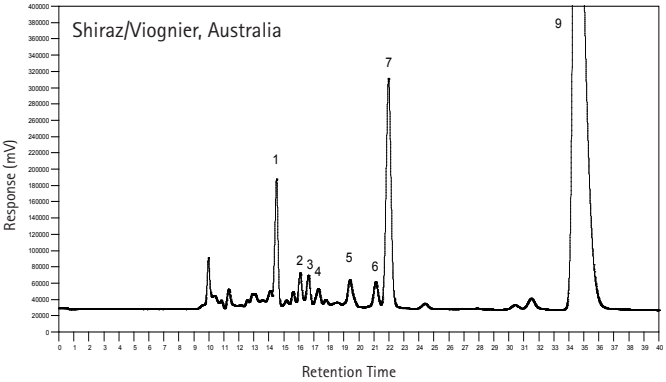
Mobile Phase: 0.004 M H_2SO_4
Flow Rate: 0.4 mL/min
Temp.: 75 °C
Detector: Varian 356-LC Refractive Index Dectector



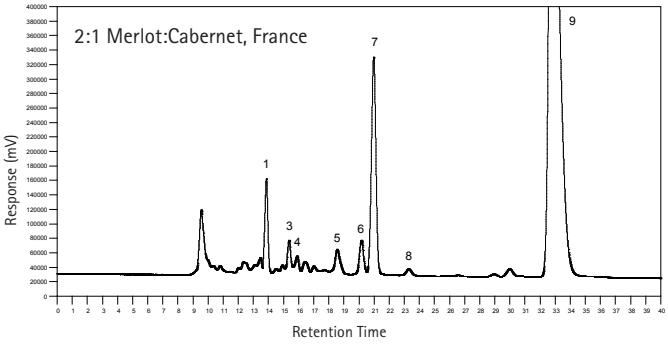
White wine contains a wide variety of organic acids and sugars.



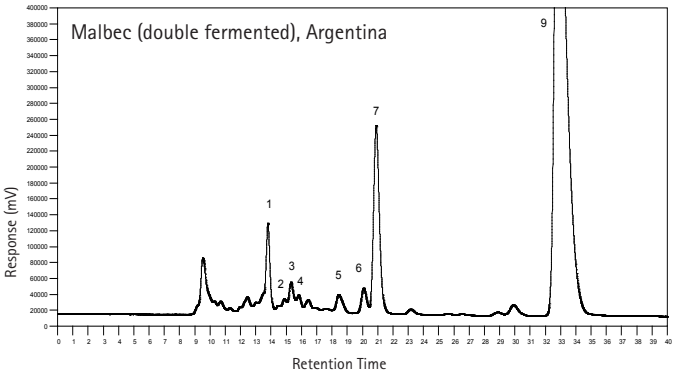
The main constituents of this red wine are tartaric acid, succinic acid and glycerol.



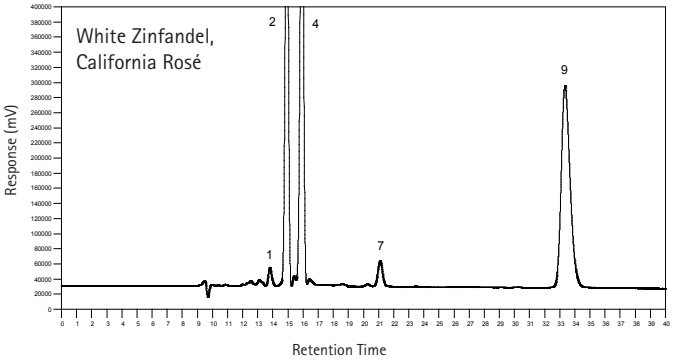
This wine contains slightly higher levels of malic acid and fructose than the Nebbiolo.



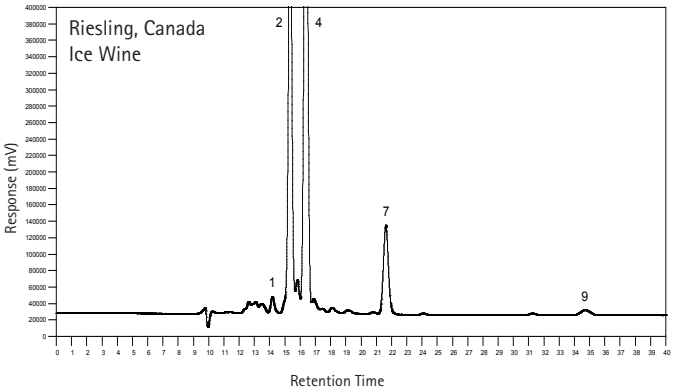
As shown, this red wine contains a slightly higher level of lactic acid than the other wines.



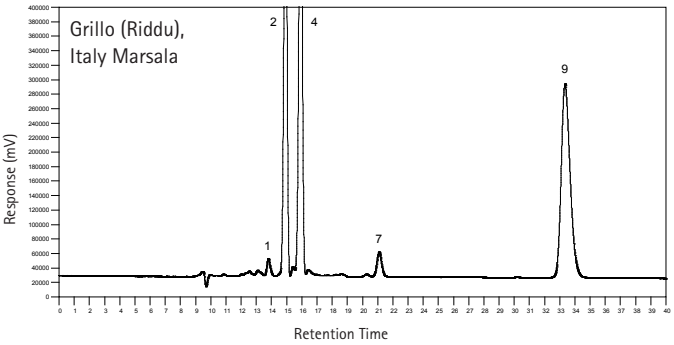
This wine has another unique profile, with differing levels of organic acids and sugar.



Rosé wine contains fewer organic acids and a significantly higher fructose content.



This dessert wine contains very high levels of malic acid, fructose and glycerol, but little else.



As expected, this dessert wine also contains high amounts of malic acid and fructose.

Key
1). Tartaric acid, 2). Malic acid, 3). Glucose, 4). Fructose, 5). Succinic acid, 6). Lactic acid, 7). Glycerol, 8). Acetic acid, 9). Ethanol

Results and Discussion

The results of this investigation prove that is possible to distinguish between different types of wine (i.e. red, white, rosé, dessert) by analyzing them via HPLC. By using an RI detector, levels of organic acids and sugars can be quantified simultaneously.

The rosé and the dessert wines all contain very high levels of malic acid and the fruit sugar fructose. In fact, Inniskillin Eiswein and Masala Wine, both dessert wines, contain up to five times as much sugar as ordinary rosé wine and 70 times as much sugar as the red and white wines. Eiswein (commonly called Ice Wine) is produced from grapes that have been frozen causing some of the water to freeze out, leaving the sugars and other solids dissolved in the remaining juice. The resulting wine is therefore very sweet but has a great deal of balancing acidity, which also explains the high level of malic acid in the wine samples.

The chromatograms of the red and white wines look very different to the others, in that they have much lower levels of sugar but much higher levels of lactic acid and glycerol. Red wine is made from the must (pulp) of red or black grapes that undergo fermentation together with the grape skins, while white wine is usually made by fermenting juice pressed from white grapes. During the fermentation process, yeast converts most of the sugars in the grape juice into ethanol and carbon dioxide, which explains the lows levels of glucose and fructose in the wine samples. Some wines also undergo malolactic fermentation, where bacteria convert malic acid into the milder lactic acid. All of these factors, and the levels of sugars and organic acids produced by the various fermentation processes contribute to the differing taste that each wine has and give each one a unique profile when analyzed by HPLC.

Some of the other peaks that appear in the chromatograms are likely to be as a result of tannins (bitter tasting plant polyphenols) present in the skins and seeds of the grapes used in the fermentation process.

Conclusion

The analysis of wines demonstrates how Hi-Plex H columns provide optimum resolution of closely eluting compounds, enabling quantitation of each. These columns are ideal for the analysis of sugar alcohols and sugar molecules using water as the mobile phase. The Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. By using the columns at higher operating temperatures, closely eluting compounds can be resolved.

References

Ding, M.-Y., Koizumi, H. and Suzuki, Y. (1995) Comparison of three chromatographic systems for determination of organic acids in wine. Analytical Sciences, **11**, 239-243.

Schneider, A., Gerbi, V. and Redoglia, M. (1987) A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. American Journal of Enology and Viticulture, **38** (2), 151-155.

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Fast Analysis of Carbohydrates in Chocolate using Ligand Exchange Chromatography with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Methods of detection for carbohydrates are severely limited as they do not normally possess chromophores or fluorophores. Detection can sometimes be accomplished in the low UV range, 190-200 nm, but unless high purity eluents are used and extensive sample preparation employed, excessive interference from other compounds may occur.

The refractive index detector is routinely used but RI is relatively insensitive, relying on a refractive index difference between solute and eluent. Where increased sensitivity is required, a pulsed amperometric detector is employed, but for uniform response, the carbohydrate must be in a high pH environment.

A better detector for the analysis of carbohydrates is the PL-ELS 1000 evaporative light scattering detector from Polymer Laboratories, now a part of Varian, Inc. When the PL-ELS 1000 is used in combination with Hi-Plex ligand exchange columns, alos from Polymer Laboratories, rapid isocratic separations of mono-, di- and oligosaccharides are achieved. The PL-ELS 1000 does not require the solutes of interest to have any particular optical properties. The principle of operation is a three stage process; the first stage involves the nebulization of the eluent, the second the evaporation of the solvent to leave solute particles and the third the detection of the light scattered by the solid solute particles as they pass through the light beam. Therefore, the only requirement for using PL-ELS 1000 is that the eluent is more volatile than the solutes.

When using Hi-Plex columns for the analysis of carbohydrates, water, with no buffer or added salt, is used as the eluent and therefore this is an ideal application for the PL-ELS 1000 detector as neutral carbohydrates have little UV activity. Sugars may be detected with the PL-ELS 1000 detector and a Hi-Plex column that has strong, cation exchange resins available in differing ionic forms.
The sulfonated column resin gives a fundamental improvement in performance and overcomes the problems of low efficiencies and high back pressures encountered with 'soft' gels. The separation mechanism is achieved initially by size exclusion, with larger oligosaccharides eluting before smaller monosaccharides, and then by ligand exchange - interaction of the numerous hydroxyl groups on the sugar molecules with the metal ion associated with the resin. Hi-Plex columns are used at elevated temperature with isocratic eluents.

Chocolate is produced in three distinct forms: dark chocolate, milk chocolate and white chocolate. The predominant sugar in the three varieties is the disaccharide sucrose. However, the milk sugar, lactose, will also be present in milk and white chocolate. The amount of lactose present will be indicative of the amount of milk solids used in the production process. As both sucrose and lactose are disaccharides, the Hi-Plex Pb column is the preferred choice for the analysis and quantification of these two components. Hi-Plex resins are available in 8% crosslinked calcium forms for the analysis of mono- and disaccharides, and hydrogen (acid) forms for the analysis of sugar alcohols and organic acids. Also available is a 4% crosslinked sodium form for the separation of high molecular weight oligosaccharides, such as corn syrups, to Dp 14.

Instrumentation

Column: Hi-Plex Pb, 300 x 7.7 mm
Detector: PL-ELS 1000 (neb=80 °C, evap=75 °C, gas=1.0 SLM)

Materials and Reagents

Eluent: Water

Sample Preparation

Aqueous solutions were prepared at a concentration of 100 mg chocolate/mL and 2 µL injection volumes were used for the quantitation.

Conditions

Flow Rate: 0.6 mL/min

Results and Discussion

Table 1 summarizes the quantitation of the two disaccharides, sucrose and lactose. Sucrose is present in all four samples with the plain chocolate having the highest level. Lactose, the milk sugar, can be seen in the other three samples. Differences in the sucrose and lactose content of the two milk chocolate samples from different manufacturers are evident.

Table 1. Disaccharide content of commercial chocolate samples expressed as a percentage by weight of chocolate.

Carbohydrate	Milk Sample 1	Milk Sample 2	Plain	White
Lactose	7	17	nd	9
Sucrose	30	41	69	42
Total	37	58	69	51

Nd - not detected

The disaccharide composition of four commercial chocolate samples is shown in Figure 1.

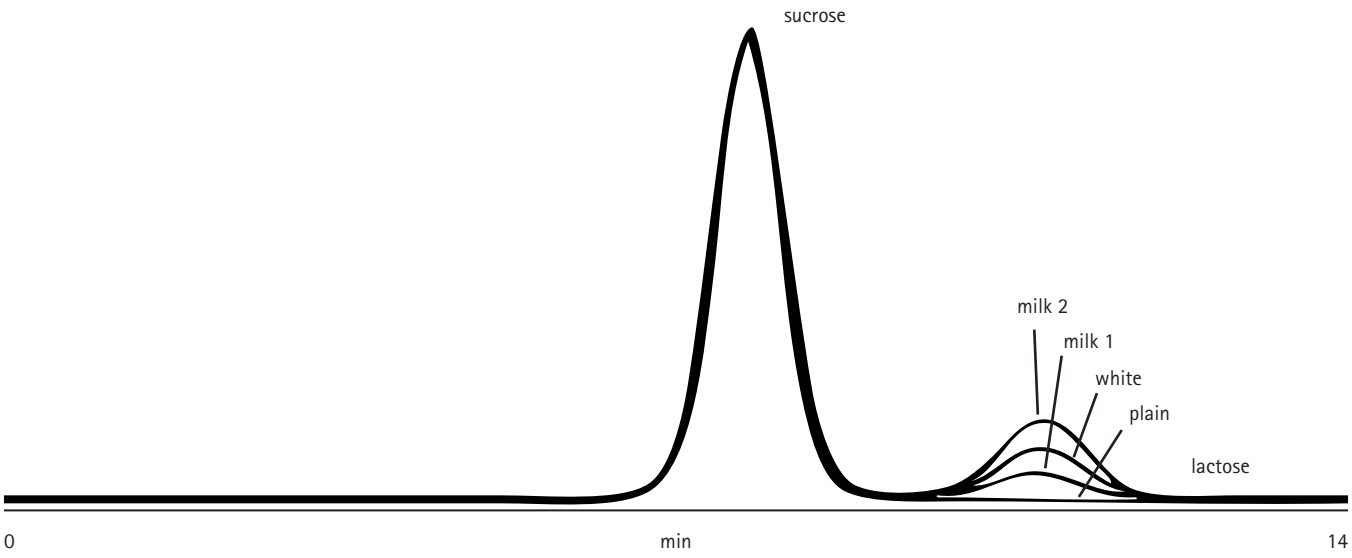

Figure 1. HPLC chromatograms of four commercial samples of chocolate, normalized to the height of the sucrose peak.

Conclusion

The composition of chocolate and levels of added milk solids in milk and white chocolate is readily achieved using water as the mobile phase with a Hi-Plex Pb column and the PL-ELS 1000 evaporative light scattering detector. The robust design of PL-ELS 1000 allows the nebulizer and evaporator to operate at very high temperatures, efficiently handling the high boiling point solvents that other ELSDs simply cannot manage.

This system avoids the use, high cost and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, Hi-Plex stays active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes compared to amino silica columns.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Determination of Melamine, Ammeline, Ammelide and Cyanuric Acid in Infant Milk-Based Formula and Other Food and Feed Products Using the Varian 220-MS Ion Trap GC/MS/MS and V:Results™ GC/MS Software

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Varian, Inc.

Introduction

In 2007, several pet food manufacturers recalled their products after finding melamine contamination, which caused serious illnesses in animals that consumed the food. In a follow-up investigation, the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) found melamine and its analogs, cyanuric acid, ammeline and ammelide (Figure 1), in various food and feed ingredients, including bakery meal, pet food, swine, poultry and fish feed[1]. In September 2008, it was reported that milk products, especially infant formula, were contaminated with melamine in China. The melamine sickened at least 15,000 infants across the country and killed at least four. Allegedly, melamine was added to the milk formula and other vegetable protein products, such as wheat gluten and rice protein, to artificially increase the apparent protein levels due to its high nitrogen content. Although melamine itself may have low or no toxicity, it is believed that melamine and its analogs form insoluble crystals in urine, causing kidney stones and eventual acute renal failure[2].

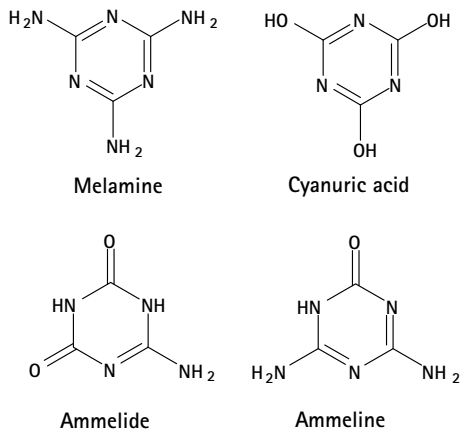

Figure 1. Melamine and related analogs.

Consequently, the US FDA developed a GC/MS method for screening and confirmation of melamine and its related analogs[1]. However, the method was not evaluated for quantitative analysis. In this note, we evaluated and developed a method using the Varian 220-MS ion trap mass spectrometer (Figure 2) to determine melamine and its analogs qualitatively and quantitatively based on the framework of the original FDA method.

Instrumentation

- Varian 220-MS Ion Trap Mass Spectrometer
- 431-GC gas chromatograph
- 8400 Trap AutoSampler
- Pierce Reacti-Therm/Reacti-Vap sample preparation system
- V:Results GC/MS software

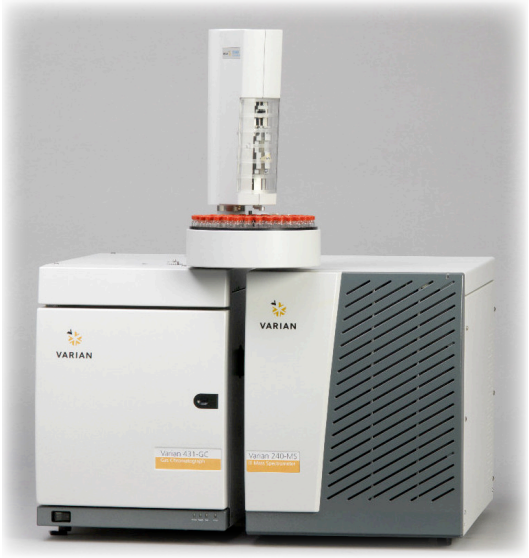

Figure 2. Varian 220-MS ion trap mass spectrometer with 431-GC gas chromatograph and 8400 AutoSampler.

Methods and Materials

Diethylamine (DEA) and pyridine (Sigma-Aldrich Co.)
Acetonitrile (Acros)
Extraction solvent: 10:40:50 DEA/water/acetonitrile

Sylating reagent: BSTFA with 1% TMCS:bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Supelco)
Melamine and cyanuric acid (Sigma-Aldrich Co.)
Ammelide and ammeline (TCA America)
Dry dog food, cat food, and infant milk- based formula from a local supermarket.

Extraction Procedure

Approximately 0.5 g of a representative sample was weighed into a scintillation vial and extracted with 10 mL of extraction solvent (10:40:50 DEA/H_2O/ acetonitrile). The sample was mixed thoroughly, sonicated for 30 min, and centrifuged for 10-30 min at 15,000 rpm. The supernatant fluid was filtered using a 0.45 µm membrane.

Trimethylsiloxane (TMS) Derivatives

Transfer 200 µL filtrate from previous step to a 2 mL vial; evaporate to dryness at 70 °C with a low flow stream of dry nitrogen using the sample preparation system. Add 200 µL of pyridine and 200 µL BSTFA with 1% TMCS to the GC vial. Vortex to mix and incubate at 70 °C for 45 min.

Standard Curve

Prepare a stock solution containing melamine and its analogs (cyanuric acid, ammelide and ammeline) at 250 µg/mL 20:80 (v/v) in a mixture of DEA/H_2O. Dilute the stock solution to prepare calibration standards at 0.04, 0.1, 0.4, 1, 4, and 10 ppm in 20:80 (v/v) DEA/H_2O. Transfer 200 µL of each individual standard into in a 2.0 mL GC vial. Follow the same TMS-derivatization procedure as used for sample preparation in pyridine. Vortex to mix, and incubate at 70 °C for 45 min. The final concentration of the derivatized standards is 20, 50, 200, 500, 2000, and 5000 ppb.

GC Conditions

Column:	FactorFour™ VF-Xms, 30 m x 0.25 mm x 0.25 µm, with 5 m EZ-Guard™ (Part no. CP9018)
Inlet Temperature:	280 °C
Injection Volume:	1 µL
Carrier Gas Flow:	Helium at 1 mL/min
Injection Mode:	Splitless
Oven Program:	75 °C for 1 min to 300 °C at 15 °C/min, and hold 4 min for a total run time of 20 min

MS Conditions

Filament Delay:	6 min
Manifold Temp:	50 °C
Transfer Line Temp:	280 °C
Trap Temp:	220 °C
Emission Current:	15 µamp
Full Scan Mass Range:	100-400

MS/MS Parameters*

Time (min)	Parent ion (m/z)	Excitation storage (m/z)	Excitation amplitude (m/z)	Product ion mass range (m/z)
7.0 - 9.45	345.2	105	60	100 - 380
9.45 - 10.24	344.2	105	65	100 - 380
10.24 - 10.90	328.3	105	72	100 - 380
10.90 - 12.50	327.3	105	80	100 - 380

*Using non-resonant waveforms

Results and Discussion

Melamine and its analogs, cyanuric acid, ammelide and ammeline, were analyzed in both full scan and MS/MS operation modes. The multiple reaction monitoring (MRM) trace of the TMS derivatives of these four compounds is shown in Figure 3.

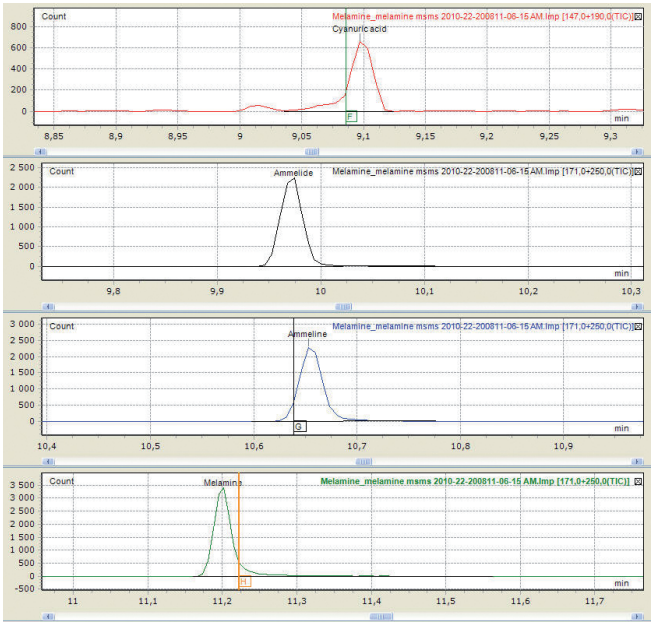

Figure 3. MRM of melamine and analogs in MS/MS operation mode at 10 ppb concentration.

This method provided excellent separation and identification of all melamine analogs. The quantitative determination of melamine was conducted from 20 to 2000 ppb in MS/MS mode. The calibration results are included in Table 1 and Figure 4. All compounds showed excellent linearity in MS/MS mode. The product ion spectra of melamine and its analogs are illustrated in Figure 5.

Table 1. Calibration of melamine and analogs.

Compound	Correlation coefficient (r^2)	Calibration range
Cyanuric acid	0.9997	20-2000
Ammelide	0.9975	20-2000
Ammeline	0.9994	20-2000
Melamine	0.9996	20-2000

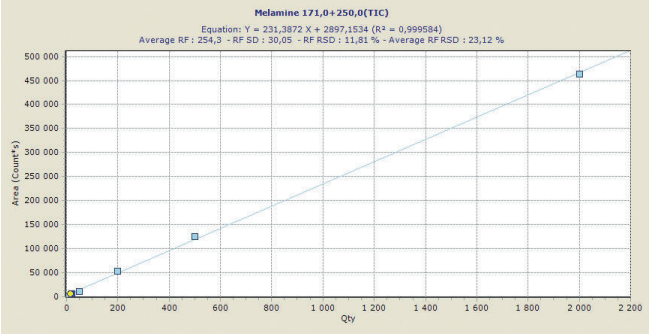

Figure 4. Calibration of melamine in MS/MS mode.

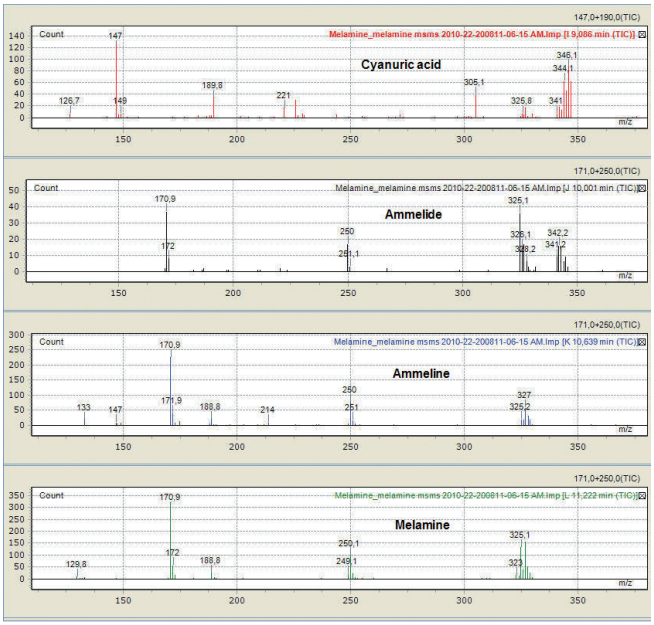

Figure 5. MS/MS product ion spectra of melamine and its analogs.

Three matrices were used to evaluate the robustness of this method. Melamine and its analogs (20 µg/g) were spiked in dry dog and cat foods and 5 µg/g was spiked in infant milk-based formula. The total ion chromatogram (TIC) of the spiked infant formula extract in full scan is shown in Figure 6. As indicated in the chromatogram, significant matrix interference was observed in full scan acquisition even when displaying the extracted ion chromatogram (EIC). In MS/MS mode, the interference from the matrix was eliminated, as shown in Figure 7 with the infant milk-based formula extract.

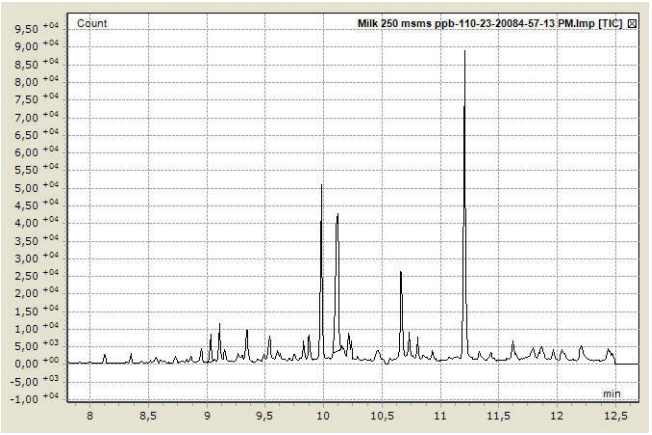

Figure 6. EIC of melamine and analogs spiked at 250 ppb in infant milk extract in full scan mode.

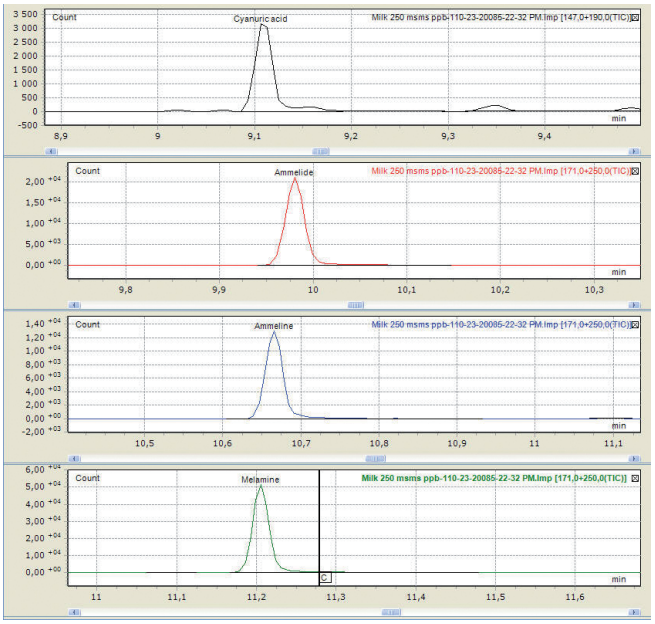

Figure 7. MRM of melamine and analogs spiked at 250 ppb in the infant formula extract in MS/MS mode.

Recovery studies of melamine, cyanuric acid, ammelide and ammeline were conducted in all three matrices at different concentrations (Table 2). Most compounds showed good recovery with excellent %RSD. Recovery of ammeline in infant milk was poorer overall with higher %RSD. This may have been due to variable extraction efficiency of ammeline from the infant milk matrix. Use of an internal standard, such as 2,6-diamino-4-chloropyrimidine, may alleviate the variation.

Table 2. Recovery and %RSD of melamine, cyanuric acid, ammelide and ammeline in MS/MS mode in dry dog food (n=3), dry cat food (n=3) and infant formula (n=6).

Average percentage recovery (%RSD)					
Matrix	Spiked (µg/g)	Cyanuric acid	Ammelide	Ammeline	Melamine
Dog food	20	132 (5)	104 (6)	102 (10)	119 (7)
Cat food	20	125 (8)	123 (9)	100 (9)	113 (8)
Infant milk	5	113 (12)	123 (8)	56 (22)	108 (6)

Conclusions

The method reported here, using the Varian 220-MS ion trap GC/MS/MS, screened and quantitated melamine and its analogs, cyanuric acid, ammelide and ammeline, in pet foods and infant formula at a concentration as low as 0.4

µg/g, which was 25 times lower than the FDA recommended level. MS/MS significantly eliminated matrix interference, providing an extra layer of confidence to positively identify and quantify target analytes. The linearity ranges studied were from 20 to 2000 ppb, and the detection limit was below 10 ppb for all four compounds.

References

1. FDA (2009) GC-MS Screen for the presence of Melamine, Ammeline, Ammelide and Cyanuric acid. US Food and Drug Administration, Silver Springs, MD, USA.
2. FDA (2009) Interim melamine and analogues safety/risk assessment. US Food and Drug Administration. US Food and Drug Administration, Silver Springs, MD, USA.

These data represent typical results.
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Application Note SI-02407

Authentication of Scotch Whisky by GC Analysis Using an Agilent J&W CP-Wax 57 CB Basic Column

Application Note

Author

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Agilent Technologies, Inc.

Introduction

The production of genuine Scotch whisky is strictly regulated to ensure its quality and to protect whisky as a premium product. Various analytical methods are employed to confirm the authenticity of Scotch whisky brands. GC analysis is one of the techniques frequently used in the identification of counterfeit whisky products.

The characteristics of Scotch whisky are strongly influenced by the cereals used in fermentation and by distillation, maturation and blending. This leads to characteristic analytical profiles for the alcohol congeners, which can be used as reference points in authenticity analysis. The CP-Wax 57 CB Basic column is highly suited for this alcohol congener identification separating the most critical peak pairs:

- 2-methyl-1-butanol and 3-methyl-1-butanol
- Isobutanol and isoamyl acetate
- Ethylacetate and acetal

Due to the intensive crosslinking of the CP-Wax 57 CB liquid phase, the column shows an excellent stability and robustness for injections of water/alcohol related samples such as spirits. The base treatment of the Wax 57 liquid phase ensures the interference free elution of furfural from acetic acid making this particular GC column highly suited for the analysis of whisky and related spirits.

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Conditions

Technique:	GC-FID
Column:	CP-Wax 57 CB Basic, 50 m x 0.25 mm x 0.20 µm (part number CP97723B)
Sample Volume:	0.5 µL
Carrier Gas:	3.0 mL/min H2, constant flow
Injector:	200 °C, split 1:25
Temperature:	35 °C. 6 °C/min, 120 °C
Detector:	FID, 210 °C

Discussion

The suspect whisky (Figure 1) exhibits a different alcohol profile compared to genuine blended Scotch whisky (Figure2). Relative concentration levels for n-propanol and isobutanol are much lower compared to a Scotch whisky, this as a result of a higher distillation efficiency used in the manufacturing process of the counterfeit product. Also the 3-methyl-1-butanol / 2-methyl-1-butanol ratio is relatively high (> 5.0) indicating a spirit of non-cereal origin (Figure 3).

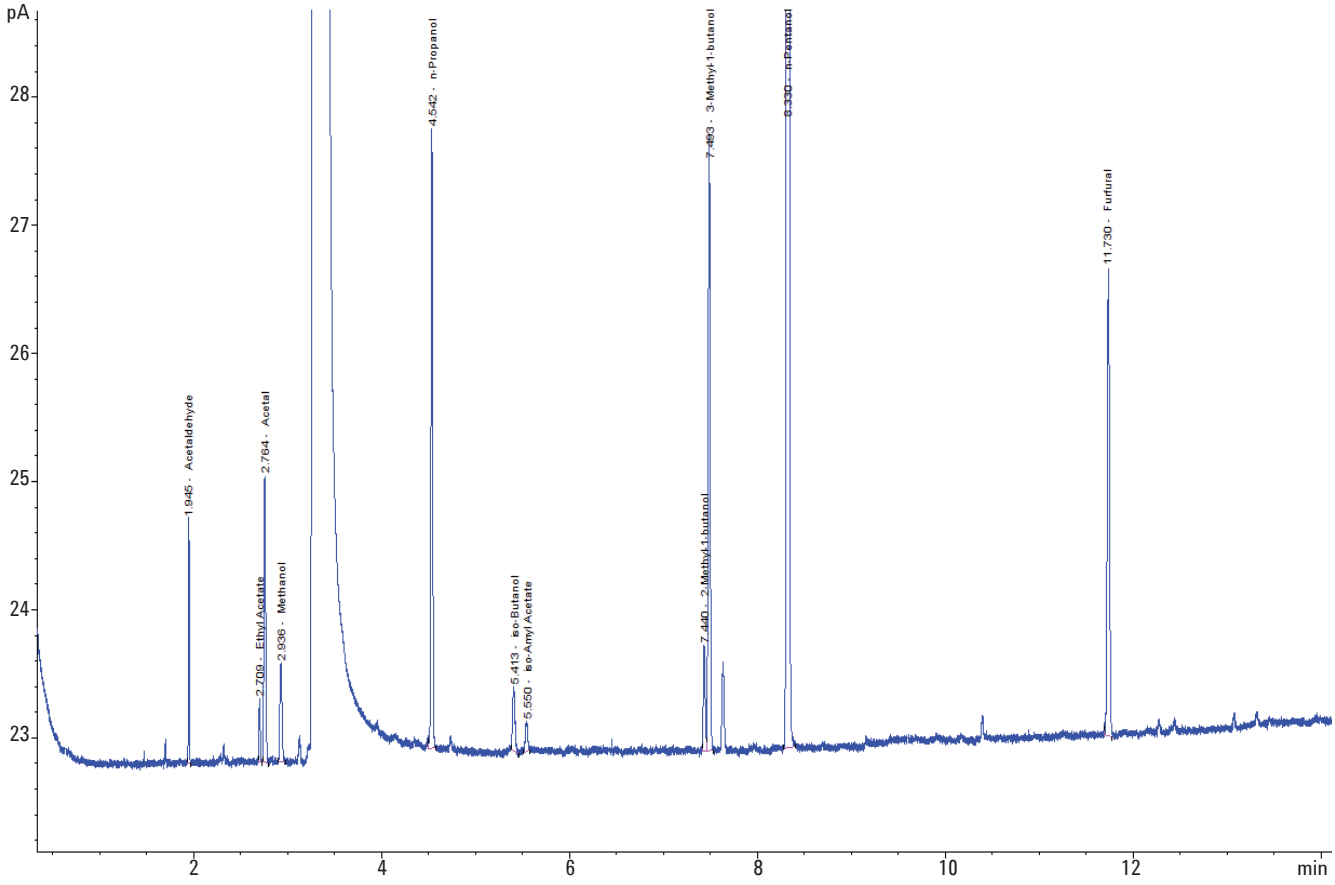

Figure 1. GC analysis of counterfeit Scotch whisky on CP-Wax 57 CB Basic

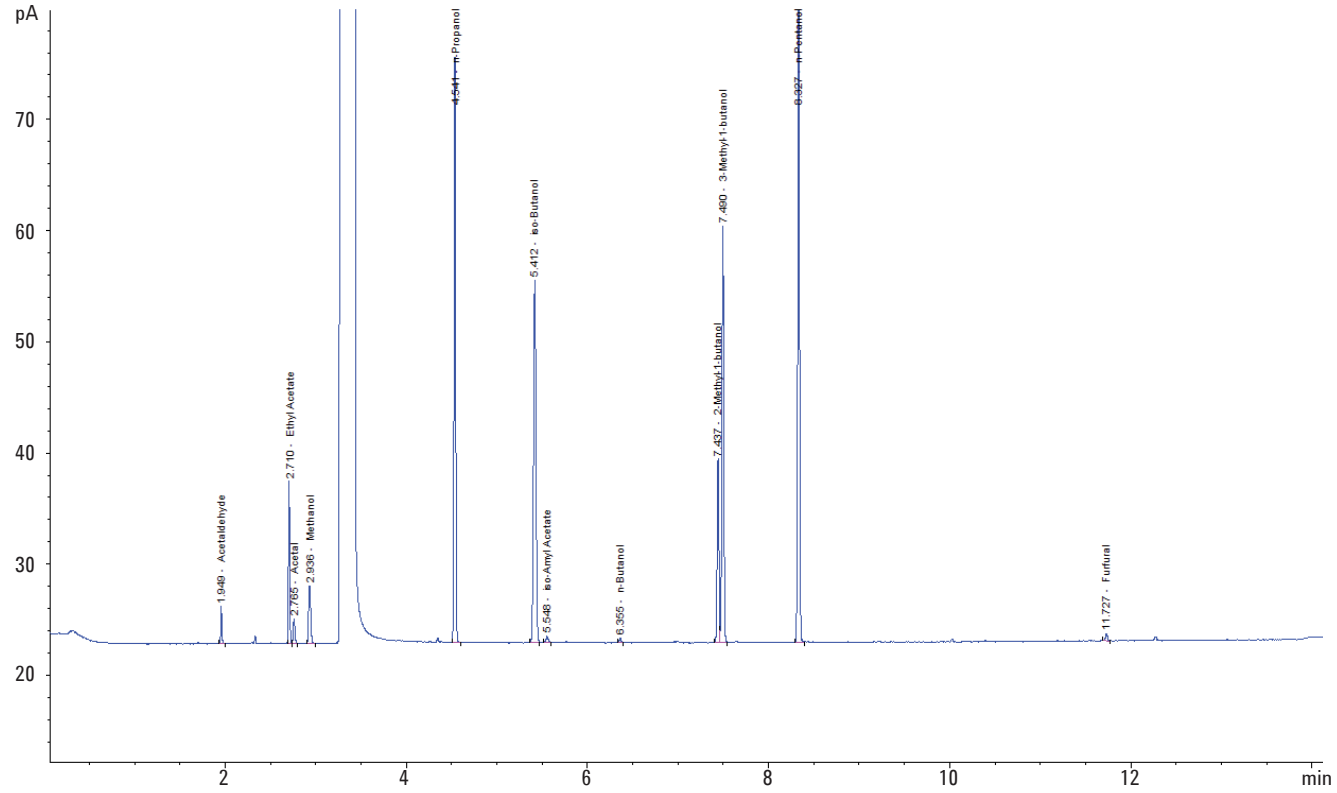

Figure 2. GC analysis of genuine blended Scotch whisky on CP-Wax 57 CB Basic

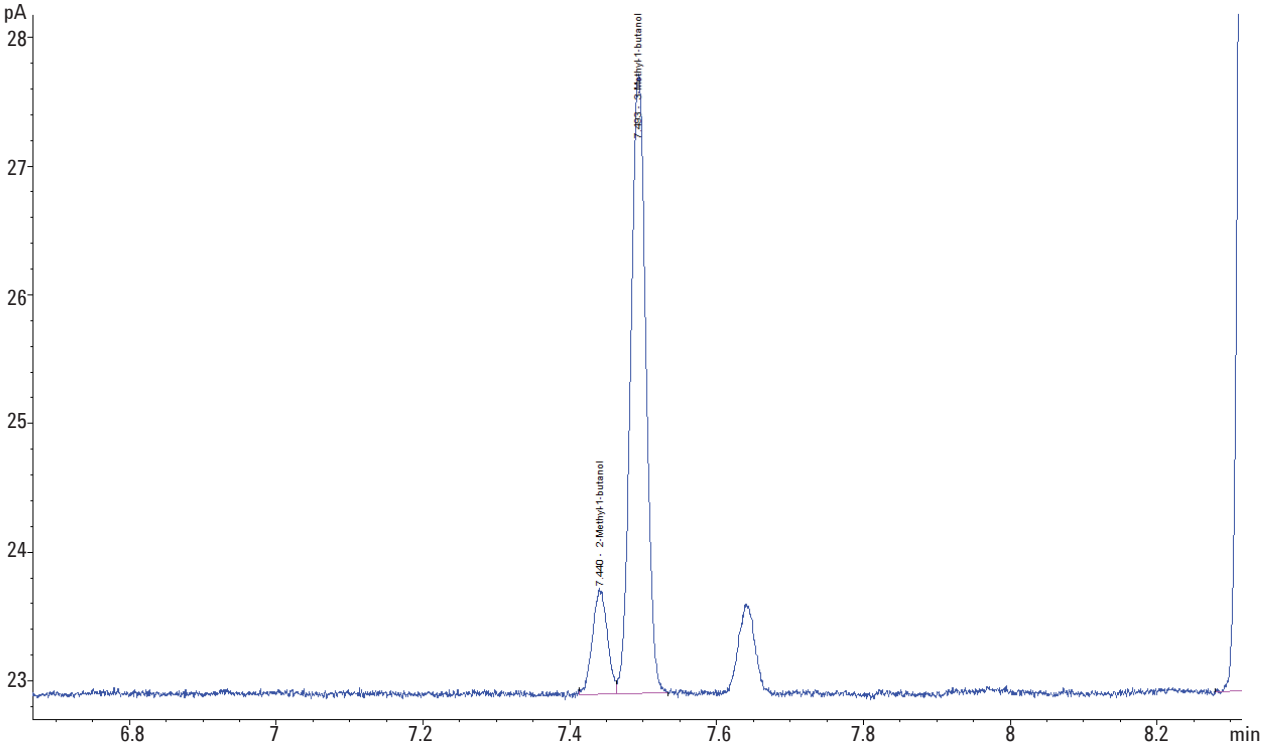

Figure 3. Expanded view of counterfeit Scotch whisky analysis on CP-Wax 57 CB Basic

Conclusions

The authentication of whisky products can be successfully carried out using the CP-Wax 57 CB Basic column because of its excellent separation of important chemical whisky descriptors such as the 2-methyl-1-butanol and 3-methyl-1-butanol. The highly durable CP-Wax 57 CB Basic column will provide a reliable GC analysis of alcoholic spirits for many hundreds of samples.

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Agilent Technologies

Development of meat speciation assays using the Agilent 2100 bioanalyzer

Application

John Dooley
Steve Garrett

Abstract

The use of real-time PCR assays for quantitative PCR is becoming more frequent. During the development of such assays it is necessary to match both the PCR primers and the fluorescent probe (used for detection) in a single reaction. The probe production costs are high compared to the primer production costs. It is, therefore, useful to know that the newly designed PCR primers are functioning in an expected manner before the cost of probe production is incurred. This Application Note describes the use of the Agilent 2100 bioanalyzer with the DNA 500 LabChip® kit to confirm that primer sets are suitable before the probe is finally produced.

Introduction

Campden & Chorleywood Food Research Association (CCFRA) is interested in the development of PCR based methods for food authenticity, particularly in relation to the detection and quantification of meat species in meat products. The assays must be applicable to processed foods and, therefore, use small DNA targets as the extracted DNA is often degraded. One approach is to develop real-time methods based on the ABI Prism 7700 Sequence Detection System, known as TaqMan. The method is suitable for amplicon detection in the range 60–150 base pairs. It is common during the TaqMan assay development stage to find several suitable probes, each with several different primer sets. Although, theoretically, these assays should all work to consistent levels, practically there are variations between them and some assays are unlikely to work at all. Therefore, it is advisable to confirm that the primers designed are specific (produce only a single PCR product with no primer-dimerization) and will work under TaqMan conditions (high $MgCl_2$ concentration and strict cycling parameters). These conditions are fundamental to the accurate quantification of samples. Although confirmation of primers can be performed using traditional agarose gel methods, the Agilent 2100 bioanalyzer has several advantages over the agarose methods including speed of analysis, accurate quantification of PCR yield and sizing of products. In addition, safety is improved as there is a reduced risk from handling DNA staining dyes such as ethidium bromide. Using the DNA 500 LabChip kit allows accurate sizing of small PCR products. This is advantageous for real-time PCR where the amplicon size required is small (less than 150 bp). We describe the use of the Agilent 2100 bioanalyzer to assist in the development of assays suitable for sensitive detection of one meat species in another.

Materials and Methods

Design of PCR assay

TaqMan PCR assays (primers and probes) were designed using the Primer Express software (Applied Biosystems, Warrington, Cheshire, UK). Primers (forward and reverse) were designed to amplify single genomic DNA targets from pig, cow, sheep, turkey and chicken of less than 150 bp, in accordance with TaqMan design restraints. Primers were produced by MWG-Biotech, UK.

Performance of PCR reaction

PCR was performed in 25-µl volumes using 300 nM of each primer, 5 mM $MgCl_2$ and 100 ng of template DNA. A TaqMan-based amplification protocol (30 cycles of a two-step reaction consisting of 95 °C for 15 seconds and 60 °C for one minute) was applied to the reactions. PCR was finished with a final 10-minute step at 72 °C.

DNA 500 LabChip preparation

Chips were primed according to Agilent's instructions, provided with the chips. Samples (1µl) of each PCR reaction were loaded onto the DNA 500 LabChip following Agilent protocols and the chips were loaded into the Agilent 2100 bioanalyzer. The analysis of the DNA products was performed using the DNA 500 protocol of the accompanying software.

Results and Discussion

To perform absolute quantification it was necessary to design two assay types, a species-specific assay and a total meat assay that would be suitable for all meat species.

Species-specific assay development

Figure 1 shows examples of results. Figure 1A was obtained following amplification of different mammal or poultry species with a specific turkey assay. A single band was obtained with turkey (lane 5) only, i.e. there was no amplification with chicken, pork,

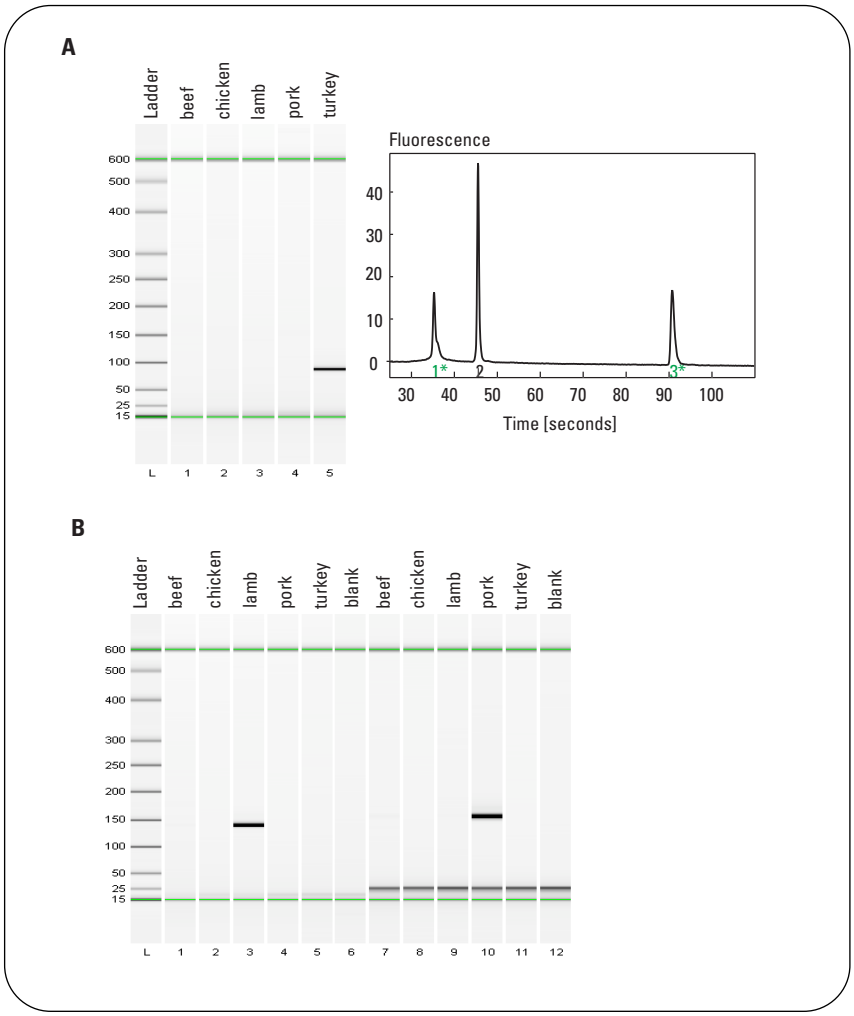

Figure 1
Species specific PCR amplification of meat samples.
A) With turkey specific primers only turkey samples are amplified. The electrophoretic trace confirms the high purity of the fragment.
B) Different sets of primers can be designed that allow the species specific PCR amplification of lamb (lanes 1-6) or pork (lanes 7-12).

beef and lamb. This band was not seen in any other poultry or mammal species. Figure 1B shows results of amplification with a set of specific mammal primers. A fragment can only be seen in the correct species, with no amplification detectable in any other species. Similar results were obtained for all five species under investigation. These results suggested that these primer sets would be suitable for individual species detection on the TaqMan. The appropriate probes were produced and the assays optimized for TaqMan usage. Species-specific amplification was observed on the TaqMan system.

Total meat assay development

Figure 2A shows the results of designing a total meat assay. The aim was to develop an assay that would amplify all meat species with the same degree of efficiency. The assay was also designed to show no amplification with non-meat species, including fish. Figure 2A shows that a single band of equal intensity was observed in all five species, whether of mammal or poultry origin. Figure 2B shows the overlay of the electrophoretic traces for these PCR products. The yields for all species were similar (mean 5.43± 0.64 ng/µl suggesting that this assay would be suitable for developing TaqMan-based, absolute quantification protocols. No amplification was observed in non-meat samples tested, including maize, soya, wheat and fish (figure 2A lanes 6-12). Figure 3 shows results from the complete TaqMan assays where the primers and probes were combined. Poor clarity (smudging) of the bands is possibly due to the use of dUTP in the TaqMan assay as opposed to the

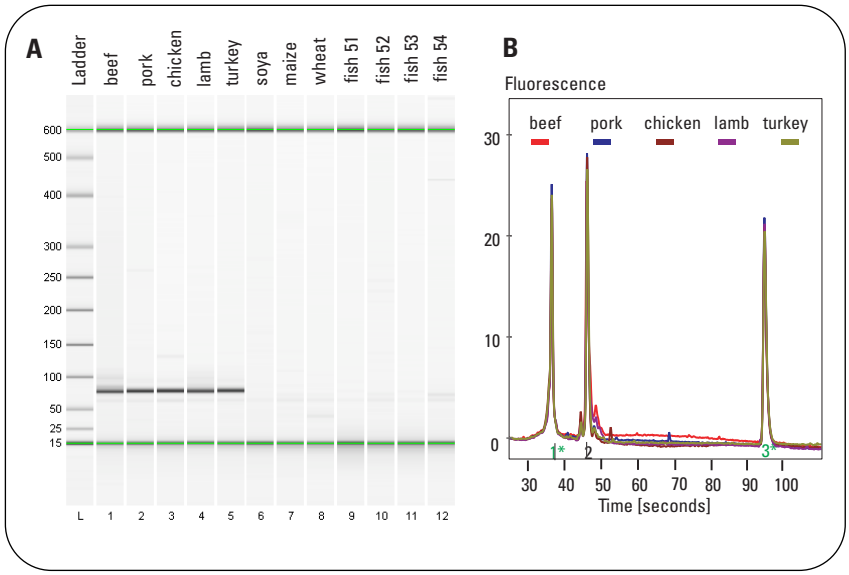

Figure 2
Development of a meat specific assay.
A) A set of primers can be designed that amplifies specifically all meat samples but does not amplify grain or fish.
B) The overlay of the electrophoretic traces reveals uniform amplification levels for different meat species.

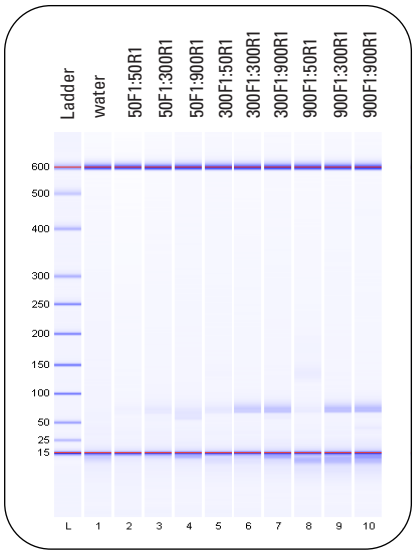

Figure 3
PCR products using TaqMan probes. Results from assy optimization test using pork DNA. Primers (forward [F] or reverse [R]) were used at 50, 300 or 900 nM concentration. Results show that at least 300 nM of F or R primer is required for amplification. 300 nM of each primer was found to be optimal for this amplification.

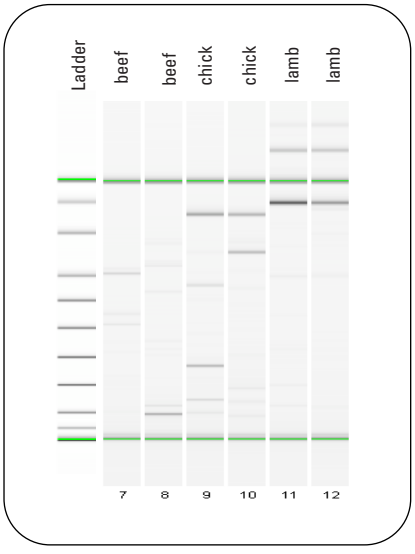

Figure 4
Non-specific amplification using non-optimal sets of primers.

use of dTTP, which was used in the conventional PCR reactions. An example of a primer set, although designed to be specific, is not specific in practice, is shown in figure 4. This assay was designed to amplify a single target in all meat species. As can be seen the number, size and yield of PCR fragments varies between the species. This primer pair was, therefore, inappropriate for use but having used the Agilent 2100 bioanalyzer to check the primers before purchasing the probe saved a considerable expense.

Conclusion

We believe the Agilent 2100 bioanalyzer provides a quick, visual method to confirm primer specificity and suitability for use in TaqMan assays. Although it would be possible to perform similar checks using SYBR Green DNA stains in the TaqMan machine itself, it is not possible to determine if the observed fluorescent change is due to primer-dimer formation or from the target of interest. The Agilent 2100 bioanalyzer allows confirmation of this and also confirmation that only a single target of expected size is being amplified. The ability of the Agilent 2100 bioanalyzer to quantify PCR yields is useful especially if assays being designed are required for quantitative or semi-quantitative determination, or as in our case to design a single assay suitable for detecting multiple species.

The authors are scientists in the Molecular Biology Group, Dept. of Chemistry and Biochemistry, Campden & Chorleywood Food Research Association (CCFRA), Chipping Campden, Gloucestershire, UK.

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Agilent Technologies

Analysis of genetically modified soya using the Agilent 2100 bioanalyzer

Application

Steve Garrett
Özge Arun
John Dooley

Abstract

In this Application Note we describe how the Agilent 2100 bioanalyzer was used to analyze multiple PCR products from Roundup Ready soya DNA. The multiplex assay assessed the effects of heat and low pH on subsequent amplification of genetically modified DNA and estimated levels of Roundup Ready soya within a sample.

Agilent Technologies

Introduction

Genetically modified organisms (GMOs) and derived food ingredients are regulated throughout the European Union (EU). Legislation requires appropriate labeling of products containing GM DNA. Whilst DNA methods based on the polymerase chain reaction (PCR) are suitable for monitoring known GMOs in raw materials and processed foods, there is concern that the analytical methods are less reliable for quantification purposes. Particular concern is for processed foods, where soya and maize ingredients, which are most likely to contain GMOs, are only a minor component of the finished product.

Food processing has a significant effect on the quality of DNA. Physical and chemical factors such as shear forces, heat treatment, nuclease activity and low pH will lead to degradation of the DNA. Soya is a common component of a wide range of foods, used as flour, protein isolate or concentrate. Soybeans are usually defatted by pressing and/or solvent extraction. In both processes, the soybeans are heated to 60–80 °C and the resulting protein meal can then be concentrated by extraction using weak acid (pH 4.5). These processes combined with further processing during product manufacture significantly reduce the quality of the soya DNA in the final product. This fragmentation of DNA reduces the probability of PCR detection particularly if the fragment sizes are smaller than the DNA sequence that is amplified by the primers.

Studies indicate that small sequences of DNA remain detectable following all but the most extreme processing conditions. In routine screening analysis for GMOs the use of small targets (<200 bp) is common. However, if there is differential degradation in these small targets (some sequences will be more susceptible to degradation than others) quantifying GMOs in processed foods using amplification of two similar but slightly differently sized targets may affect the accuracy of the results.

The aim of this project was to study the effect of food processing on PCR-based amplification and quantification of GM DNA. The approach was to develop a simple model assay system to observe differences in detection when using small targets in Roundup Ready (RR) soya heat treated at low pH. The Agilent 2100 bioanalyzer was used in post-PCR analysis to measure the concentration and number of differently sized PCR products.

Results

Assay development

The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled four GM soya targets to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate-3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya (Monsanto).

Although gel-based analysis enables sizing of PCR products, it cannot be used to provide accurate information on the quantity of a PCR product. Therefore, post-PCR analysis was performed using the Agilent 2100 bioanalyzer, which can accurately size and quantify PCR products. Initially, this was carried out using the DNA 7500 LabChip® kit. Four peaks were observed, corresponding to the PCR products within the lectin and EPSPS genes. However, the 80 bp peak from the lectin gene was not completely resolved from the alignment marker so quantification was not possible. Subsequently, post-PCR analysis was performed using the DNA 500 LabChip® when it was made available and resolution of all four peaks was observed (figure 1).

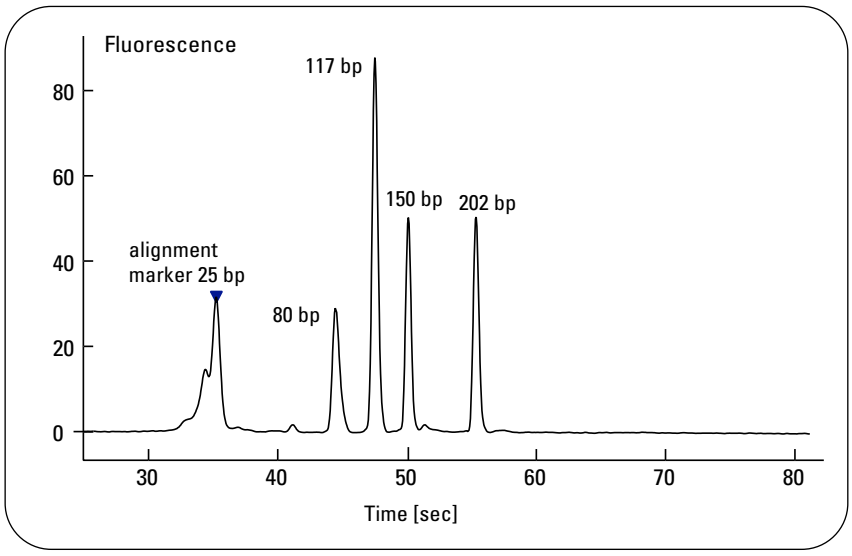

Figure 1
Multiplex assay for GM soya. Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit.

The multiplex PCR assay was applied to DNA from RR soya flour reference materials (figure 2). The results show that there is an increase in PCR product concentration of the 117 bp, 150 bp and 202 bp products and little change in the concentration in the 80 bp product. This increase corresponds to the increase in RR content of the soya flour. No increase in the product from the lectin gene was expected, as it is common to both the GM- and non-GM soya. It should therefore be possible to estimate levels of GM soya in an unknown material by applying the multiplex assay and comparing the ratio of lectin product to the other products in the sample with ratios produced from certified reference materials (CRMs). The assays would have to

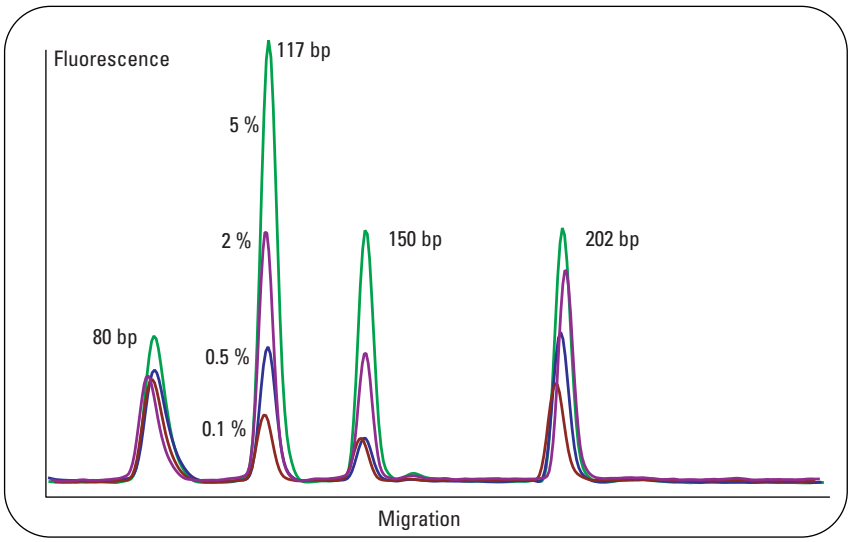

Figure 2
Peaks produced by the Agilent 2100 bioanalyzer using the multiplex assay on CRMs containing different levels of GM soya

Sample	Ratio 117/80	Ratio 150/80	Ratio 202/80
Extract 1a	1.50	0.68	1.04
Extract 1b	1.45	0.68	1.04
Extract 2a	1.40	0.46	0.71
Extract 2b	1.54	0.65	1.10
5% CRM	2.10	1.00	0.94
2% CRM	0.74	0.21	0.68
1% CRM	0.41	0.18	0.43

Table 1
Analysis of a soya flour

be performed using a limited number of PCR cycles in order to perform the end-point detection during linear stages of amplification. The reference materials and the unknown samples would also have to be similar in nature.

Analysis of a soya flour containing GM soya

The multiplex assay was applied in duplicate to two DNA extracts prepared from a soya flour sample which had given a positive result when screened for a common GM promoter sequence (CaMV 35S promoter). The assay was also applied to 1 %, 2 %, and a 5 % CRMs. Ratios of each EPSPS product compared to the lectin product were calculated (table 1). The same extracts were analyzed using a real-time PCR method for quantitative determination of GM soya. Results from the real-time analysis indicated that the sample contained approximately 5 % GM soya while the multiplex assay gave ratios indicating that the sample contained between 2 % and 5 % GM soya.

The effect of heating time and pH on detection and quantification of GM-DNA

The multiplex PCR assay was applied to soya flour samples containing approximately 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Time at 100 °C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

Table 2
The effect of heating time on RR flour held at pH 3.3, determined using the multiplex PCR method.

To eliminate any variation due to amount of DNA in each PCR reaction, the ratio of the lectin 80 bp product to each of the other three products was determined for all experiments (table 3), that is, normalized with respect to the 80 bp product. The ratios of each would be expected to remain constant if no degradation of the target DNA occurred or if the degree of degradation between the 80 bp target and the other targets was comparable. At pH 3.3 the ratios tended to increase with increasing heating time. This suggests that at low pH there were differences in the detectability of the three EPSPS targets compared to the smaller lectin target, with the

Time at 100 °C (min)		Ratio lectin 80bp/ RR-117bp	Ratio lectin 80bp/ RR-150bp	Ratio lectin 80bp/ RR-202bp
pH 3.3	0	1.8	3	1.9
	3	1.8	3	2.1
	6	1.7	7.8	17.5
	9	3	5	5
	12	3.6	4.4	6
	15	3.8	9	NP
	18	NP	NP	NP
pH 4.3	0	2	4.4	1.9
	3	2.2	2.9	1.8
	6	1.3	2	1.9
	9	1.3	2.2	2.3
	12	1.5	2.6	2.6
	15	1.8	3.7	2.7
	18	1.9	3.9	3
pH 6.7	0	1.8	4.2	1.7
	3	1.7	3.9	1.6
	6	1.2	2.3	1.5
	9	1.5	2.4	1.7
	12	1	1.9	1.3
	15	1.2	2.1	1.3
	18	1.4	2	1.4
	21	1.6	2.4	1.4

NP= no PCR products observed

Table 3
The effect of heating time on RR flour held at pH 3.3, 4.3 and 6.7, determined using the multiplex PCR method

80 bp target being degraded at a slower rate compared with the other targets. At pH 4.3, the 80/118 bp and 80/145 bp ratios decreased during the first 3–9 minutes of heating, then increased returning to the their original value, whereas the 80/202 bp ratio increased with heating time. Similar trends were observed at pH 6.7 except for the 80/202 bp ratio where little change occurred. However, further analyses are required to replicate these observations and focus around the pH where an effect is observed. These initial results indicate that the different targets used in PCR are not detected equally in these experiments.

Other studies show similar results. In 1998 Hüpfer et al.[1] demonstrated that PCR detection of GM maize in polenta could be influenced by pH during thermal treatment of the product. They showed that detection of a 1,914 bp segment of the cry1A(b) gene was not possible after boiling at neutral pH for 30 minutes, whereas a 211 bp fragment was detected after boiling for 105 minutes. At pH 2-3, the larger segment was not detected after boiling for 5 minutes and the smaller fragment was not detected after 15 minutes. As a result of such observations, it is common practice to use small target sequences in screening methods for GMOs. However, the work reported here suggests that at low pH, degradation of DNA results in differences in detection of very small target sequences. This may not be important for qualitative analysis, however, it is likely to have significance for the accuracy of quantitative analysis of processed foods with low levels of GM-DNA, when two target sequences are analyzed simultaneously as in real-time PCR.

Conclusion

The RR multiplex assay was used to quantify the amount of GM soya in a soya flour and assess the effects of pH and heat on the detection of GM soya DNA. A key component of the assay is the Agilent 2100 bioanalyzer which is used to accurately quantify the four PCR products simultaneously. This user-friendly instrument replaces gel based analysis and offers enormous potential for the routine screening of raw materials for levels of genetically modified organisms.

References

1.
Hüpfer C, Hotzel H., Sachse K., Engelk E.H. “Detection of the genetic modification in heat treated products of Btmaize by polymerase” *Zeitschrift für Lebensmittel Untersuchung und Forschung A.*, *206*, *203-201*, **1998**

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Agilent Technologies

Detecting genetically modified organisms with the Agilent 2100 bioanalyzer

Application

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Genolife

Abstract

Labeling of food containing more than 1% of genetically modified organisms (GMOs) has been obligatory in Europe since January 2000. To guarantee transparency and labeling, methods to distinguish between transgenic food and their traditional counterparts must be available. Genolife developed a method to detect Ready RoundUp soy (RRS) and a multiplex PCR to detect five corn transgenes (Bt176, Bt11, MON810, T25 and GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, high throughput and standardized way to analyze multiplex PCR products. The methods developed by Genolife allow detecting 0.01% of RRS in food ingredients on the one hand and 10 copies of transgene MON810, GA21 and Bt11, and 100 copies of transgene Bt176 and T25 on the other.

Agilent Technologies

caliper LabChip®

Introduction

The rapid development of biotechnology has launched products and ingredients derived from genetically modified organisms (GMOs) into the food market. The general public, however, has shown anxiety about this new technology. Information and transparency regarding these products are essential in order to become accepted by the consumers. In Europe, labeling of GMOs is regulated by the Novel Food directives 258/97/EEC[1] and 1139/98/EEC[2]. More recently, the "threshold regulation" 49/2000/EEC[3] has been approved, specifying that foodstuffs are subject to labeling. When the proportion of an individual food component is higher than 1 % manufacturers must label their products. Moreover, the presence of GMOs must be adventitious and therefore, food manufacturers must be able to supply evidence that they have taken appropriate steps to avoid using GMOs. A key factor to guarantee transparency and labeling is the availability of methods to distinguish between transgenic food and their traditional counterparts, not only in raw materials but also in food products.

Several analytical methods using polymerase chain reaction (PCR) technology have been developed to qualitatively detect the presence of a modified sequence of nucleic acid in transgenic food.[4,5] But these analytical methods detect only one modified sequence of one genetically modified organism. It would be advantageous to detect more than one sequence per genetically modified organism (one endogenous gene and several transgenic markers) or to screen several GMOs in one analysis. We therefore developed a method to detect RoundUp Ready soy (RRS) from Monsanto in one part, and another method to detect one endogenous maize gene and five genetically modified maize genes – four are authorized in Europe (Bt176, Bt11, MON810 and T25) and one is non-authorized (GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, rapid and standardized alternative to analyze multiplex PCR products.

Results and discussion

RRS detection in food ingredients

DNA was extracted from commercial transgenic soybean reference standards (Fluka) and different food samples (lecithin, soybean proteins, soybean flour) with specific protocol developed by Genolife. For each sample, four PCR reactions were done — one for amplification of an endogenous gene (ACC1, 115 bp) to check the quality of the extracted DNA, and three PCR reactions for specific RR soybean sequences (T1: 167 bp, T2: 141 bp and T3: 189 bp). After amplification, PCR products were mixed and 1 µl of each mixed PCR was analyzed on the Agilent 2100 bioanalyzer using the DNA 500 LabChip kit, which allows analysis of DNA fragments ranging in size from 25 to 500 bp. Twelve samples were analyzed simultaneously and the 2100 bioanalyzer produced raw data and analysis in multiple formats. It displayed a simulated gel view an electropherogram. A data table labels each of the peaks and furnishes information about the size and concentration for each fragment. Results are shown in figure 1. Non transgenic soy gives

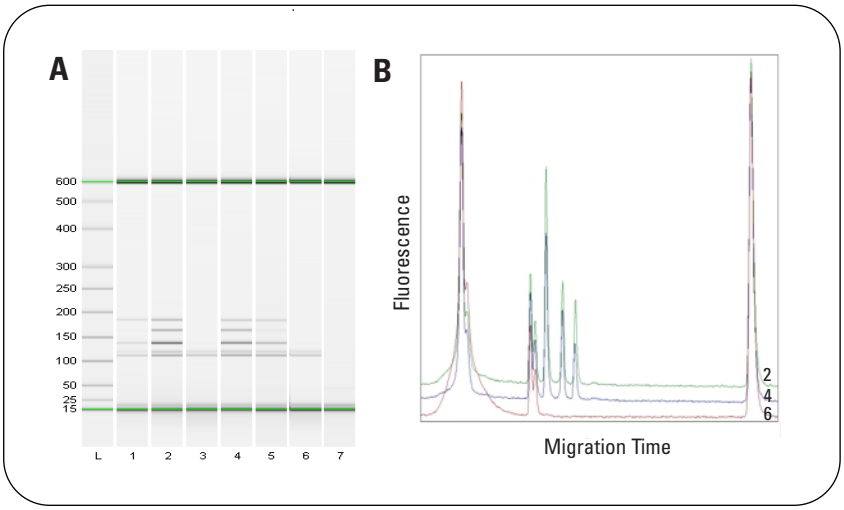

Figure 1
RR soybean detection
A) Gel view: 1-soya protein, 2- lecithin, 3-soybean flour, 4-RR soy 1 %, 5-RR soy 0.1 %, 6-non transgenic soy, 7-PCR blank.
B) Overlay of the electrophoretic traces of lanes 2, 4 and 6.

one band corresponding to the endogenous gene (115 bp) while RR soy 1 % and RR soy 0.1 % show four bands corresponding to the endogenous gene (115 bp) and the three specific RR soybean sequences (141, 167 and 189 bp). The Agilent 2100 bioanalyzer software compares unknown samples with commercial transgenic soybean reference standards. Soybean proteins and lecithin are transgenic (four bands at 115, 141, 167 and 189 bp) and soybean flour is not transgenic (only one band at 115 bp corresponding to endogenous gene). The 2100 bioanalyzer performs quantification using an internal standard (marker) added to each sample before loading, and the software calculates the DNA concentration in each band. Soybean protein contains less than 0.1 % and lecithin more than 1 % RR soy, compared to concentration DNA in transgenic band obtained with commercial transgenic soybean reference standards (table 1). The detection limit of this RR soy PCR is 0.01 %.

	RR1 %	RR 0.1 %	Soya protein	Lecithin
141 bp	1	0.24	0.12	1.3
167 bp	0.1	-	-	0.34
189 bp	1	0.56	0.48	1.3

Table 1
DNA concentration (ng/µl) of the band corresponding to the transgenic markers

GMO maize detection in food ingredients by multiplex PCR

In Europe, four GMOs maize were authorized — two insect-resistant corn species from Novartis (Bt11 and Bt176), one insect-resistant corn from Monsanto (MON810) and one glufosinate-tolerant corn developed by Agrevo (T25). We developed a PCR multiplex to detect these four corn lines and one endogenous gene to check the integrity of the extracted DNA. We also added a couple of primers to detect a glyphosate-tolerant corn GA21 produced by Monsanto. This glyphosate-tolerant corn is authorized in the USA and can be exported in Europe with authorized corn. The PCR multiplex amplified a 152-bp fragment for endogenous gene, a 343-bp fragment for Bt176 corn, a 149-bp fragment for T25, a 199-bp fragment for MON810, a 110-bp fragment for Bt11 and a 270-bp fragment for GA21. Results are presented in figure 2. The endogenous gene was amplified in all lanes except PCR blank. Only Bt176 fragment (343 bp) was obtained when only Bt176 corn was present in PCR tube (lane 4). Specificity was checked for each corn (lane 5: Bt11, lane 6: T25, lane 7: GA21 and lane 8: MON810). Lanes 1 to 3 presented PCR products when all corn lines were analyzed together. This multiplex PCR allows detection of five corn lines present at 0.2 % each (lane 3). The detection limit of this multiplex PCR is 10 copies for transgene MON810, GA21 and Bt11 and 100 copies of transgene Bt176 and T25.

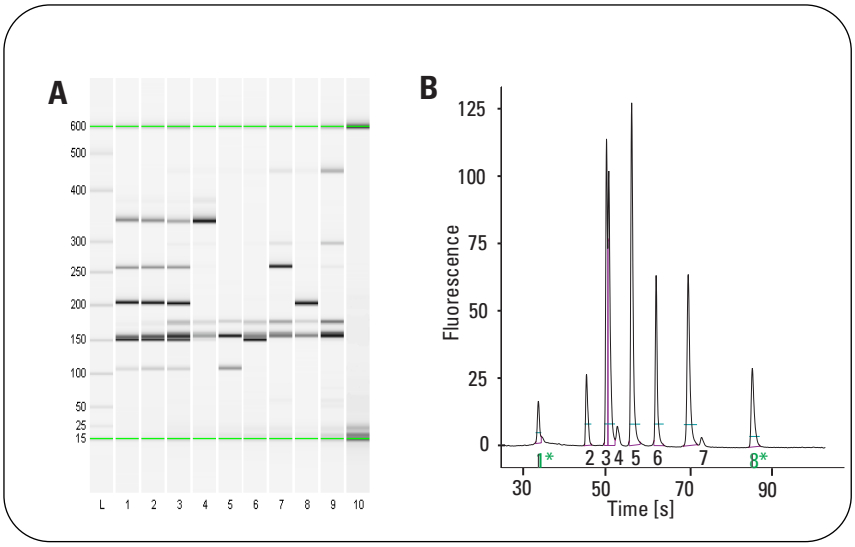

Figure 2
Multiplex PCR to detect GMO corn
A) 1-Bt176 5 %, Bt11 2 %, T25 5 %, GA21 5 %, MON810 5%, 2-Bt176 2 %, Bt11 2 %, T25 2 %, GA21 2 %, MON810 2 %, 3-Bt176 0.2 %, Bt11 0.2%, T25 0.2 %, GA21 0.3 %, MON810 0.3%, 4-Bt176 2 %, 5-Bt11 2 %, 6-T25 2 %, 7-GA21 2 %, 8-MON810 2 %, 9-non GMO corn, 10-PCR blanc
B) Electrophoregram of lane 2 (multiplex PCR with mix of 2 % corn). The peaks are: Bt11 corn (2), T25 corn (3), endogenous gene (4), MON810 corn (5), GA21 corn (6) and BT176 corn (7).

Conclusion

We developed detection methods for GMO soy and corn in food ingredients. The Agilent 2100 bioanalyzer performed the analysis of multiplex PCR product and allowed a semi quantification of GMO content. Two detection methods were presented— one for RR soy and one for GMO corn detection. The sensibility of the soya detection method is 0.01 % and 10 to 100 copies of transgene for corn mulitplex detection.

References

1.
Regulation (EC) No 258/97 of the European Parliament and the Council of 27 January 1997 concerning novel foods and novel food ingredients. *Official Journal L 043*, 14/02/1997 p.0001-0007.

2.
Council Regulation (EC) No 1139/98 of 26 May 1998 concerning the compulsory indication of the labeling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC. *Official Journal L 159*, 03/06/1998 p.0004-0007.

3.
Commission Regulation (EC) No 49/2000 of 10 January 2000 amending Council Regulation (EC) No 1139/98 concerning the compulsory indication on the labeling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC. *Official Journal L 006*, 11/01/2000 p.0013-0014.

4.
Vaïtilingom M., Pijnenburg H., Gendre F., Brignon P. (1999) Real-Time Quantitative PCR Detection of Genetically Modified Maximizer Maize and Roundup Ready Soybean in some representative Foods. *J. Agric. Food Chem.*, 47, 5261-5266.

5.
Zimmermann A., Hemmer W., Liniger M., Lüthy J., Pauli U. (1998) A sensitive Detection Method for Genetically Modified MaisGardä Corn using a Nested PCR-system. Lebensm.*Wiss.u.-Technol.*, 31, 664-667.

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Agilent Technologies

Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 Bioanalyzer

Application

Food

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Abstract

This application note describes how the Agilent Technologies 2100 Bioanalyzer can be used to analyze protein extracts from transgenic seedlines. Accuracy and precision in the determination of protein size and concentration was sufficiently good to allow for the characterization of experimental seedlines based solely on expressed protein profiles.

Introduction

ß-conglycinin(7S) and glycininin(11S) are the primary seed storage proteins in soybean, comprising about 70% of the total storage proteins. Because these proteins make up such a large portion of soya protein, they are of critical economic importance. Characterizing the expression of these proteins in various soybean seed lines is also essential in expanding the range of soy protein applications in food. The relative levels of these two proteins have been shown to significantly impact the nutrition, taste, and texture of food products derived from soy protein extracts [1]. For this reason, soybean lines that preferentially express the 11S or 7S proteins continue to be an active target in the efforts to improve seed quality.

Both conglycinin and glycinin are complex aggregates made of smaller protein subunits. β-conglycinin is a 7S protein with a trimeric structure and is composed of 53, 70, and 76 kDa units. Glycinin is an 11S hexameric protein consisting of six monomer units, where each monomer is made up of 40 and 20 kDa subunits [2].

Given the sizes of protein subunits, it is relatively straightforward to characterize the levels of these two proteins by electrophoresis. The Agilent 2100 Bioanalyzer, an automated microfluidic electrophoresis platform, is well suited for the analysis of proteins in this size range. The Protein 200 LabChip has a size range of 14-200 kDa. Samples of soy protein isolate can be loaded, separated, and analyzed for relative protein composition in less than 45 minutes. In this application we describe the use of the Agilent 2100 Bioanalyzer in the analysis of soybeans, to determine the level of expression of 7S and 11S proteins.

Experimental

Extraction Protocol

Grind the seed into a fine powder. Place a 30 mg sample in an Eppendorf® tube and add 1000 µL of extraction buffer (50 mM Tris [pH 7.5], 10 mM 2-mercapto-ethanol, 0.1% SDS). Agitate the mixture on a rotary shaker for 30 minutes and then centrifuge at 15,000 g for 10 minutes. Remove the supernatant and introduce the extract directly into the Protein 200 LabChip to begin the assay protocol.

If the extracts contain an excessive amount of oil, the supernatant may be removed and further diluted prior to beginning the Protein 200 protocol.

Methodology

To determine if a transgenic line of soybeans preferentially expressed the β-conglycinin or glycinin protein groups, seed extracts were compared to extracts made from wild-type seed lines that strongly expressed either the 7S or the 11S group. Twenty extracts from the unknown seedline were prepared as described above. The protein profiles were determined by separating the proteins in the Agilent 2100 Bioanalyzer. Because of the size range of the proteins, the Protein 200 Plus chip (14-200 kDa) was used for the separation. The concentration of the individual proteins was determined by a comparison with an internal concentration marker (myosin). The ratio of 7S/11S proteins was then calculated from those values and the ratio was compared to the ratio of wild-type seedlines that preferentially expressed either 7S or 11S protein groups. Figure 1 shows the electropherogram for a control seedline, a 7S wild-type, an 11S wild type, and a representative sample from the unknown transgenic seedline. A simulated gel image of the same traces is shown in Figure 2.

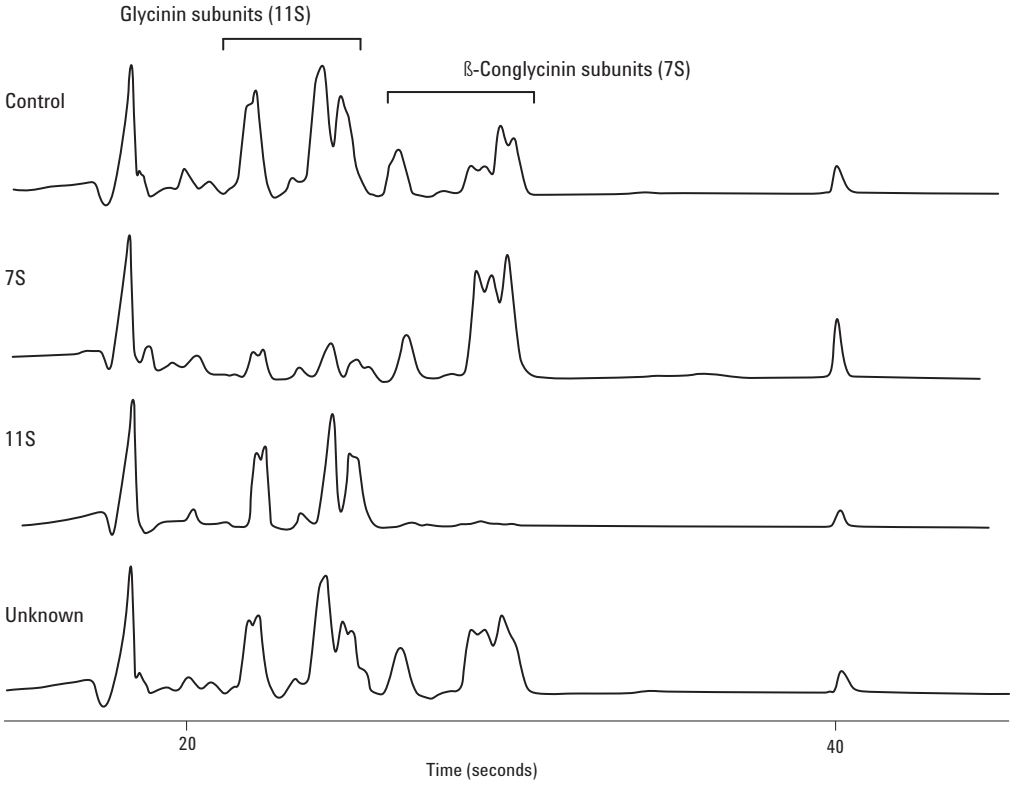

Figure 1. Electropherograms of soya protein extracts.

The ratio of 7S to 11S for the 20 sample extracts was calculated by integrating the individual components comprising the 7S and 11S groups and then determining the summed areas of the two groups. The levels of extracted protein and the 7S/11S ratios for the control 7S, 11S, and unknown groups are summarized in Table 1.

The ratios determined for the high 11S and high 7S seedlines indicate the range of expected 7S/11S ratios should fall between 0.04-3.4. The ratios determined for both the controls and unknown extracts both fall within this range. All 20 unknown samples showed a higher 7S/11S ratio than the control. Average ratio values for 20 unknown extracts and 5 control extracts was 0.72 and 0.39, respectively. Measurement precision was excellent for both sample sets.

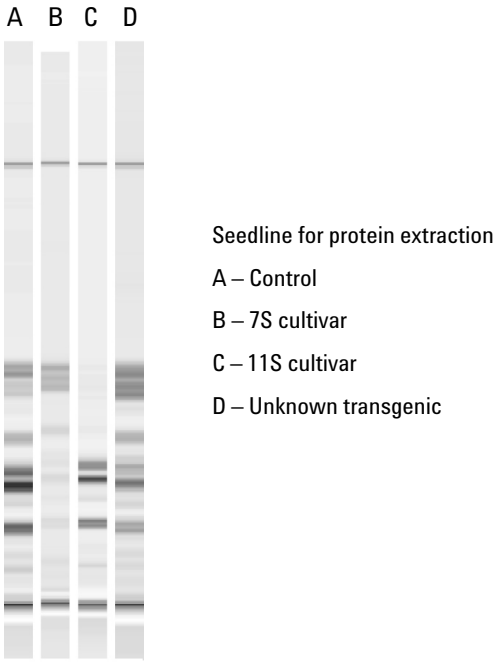

Figure 2. Gel simulation of electropherograms for soya protein extracts.

Table 1. Summary of Extracted Protein Levels and 7S/11S Protein Ratios

SeedLine	Extracted protein level µg/mL	7S/11S Ratio
Control	14,000	0.39 ±0.004 (n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknowns	13,000	0.72 ±0.1 (n=20)

Conclusions

This application note describes the use of the Agilent 2100 Bioanalyzer and the Protein 200 LabChip Kit for evaluating the relative expression of β-conglycinin and glycininin in unknown seedlines. In the 20 protein extracts taken from the unknown seedline, the average ratio was 0.72 ±0.1. This ratio lays in range that is characteristic of high 7S expression seedlines. Given the precision of the ratio determination, it is clearly apparent that the assignment of this unknown seedline to the high 7S group is statistically significant. This conclusion is further supported by a comparison to a normal control seedline where the ratio of 7S/11S is 0.39 ±0.004.

The Agilent 2100 Bioanalyzer together with the Protein 200 LabChip Kit are quick and efficient tools for the determination of relative protein expression. The resulting protein expression profiles are in turn a highly effective means for the characterization of new transgenic seedlines.

References:

1. Kitamura, K., (1995) "Genetic Improvement of Nutritional and Food Processing Quality in Soybean," *Jpn. Agric. Res. Quart.*, **29(1)**, 1-8.
2. Yaklich, R. W., (2001) "-Conglycinin and Glycinin in High-Protein Soybean Seeds," *J. Agric. Food Chem.* **49**, 729-735.

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Agilent Technologies

Nested Multiplex Polymerase Chain Reaction for the Determination of DNA From Genetically Modified Corn and Soy Beans Using the Agilent 2100 Bioanalyzer

Application

Agriculture

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Abstract

This application note describes how the Agilent Technologies 2100 bioanalyzer and the DNA 500 LabChip can be used to detect polymerase chain reaction products corresponding to genetically modified elements and endogenous sequences in corn and soy beans. The DNA extraction protocol used in the preparation of polymerase chain reaction samples was characterized using the Protein 200 Plus LabChip.

Introduction

Six years after the introduction of genetically modified organisms (GMO), consumer concerns about the presence of such modified organisms in food remains an ongoing issue. There has been a continuous debate surrounding issues of how food products that contain GMO ingredients should be regulated and labeled. This debate has been further complicated by disagreements over how GMOs should be detected and the significance of the detected levels. In spite of these issues, the number of available transgenic events has continued to grow. At the current time there are 14 transgenic varieties of corn and soy beans that have been deregulated by the Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA).

Although enzyme immunoassay is an efficient means for detecting transgenic proteins in raw products, only DNA analysis has proved to be effective for the entire range of sample matrices from raw materials to highly processed foods. The polymerase chain reaction (PCR) has been widely accepted as a method for the detection of DNA from genetically modified ingredients such as soya or corn [1, 2]. PCR detection of GMOs can be done either as a screening test using endpoint PCR or as a quantitative test using real-time fluorescence detection of the PCR product.

Quantitation by real-time PCR is an expensive analysis requiring assay calibration for each sample lot and multiple replicates of each unknown sample. Typical service charges for a single analysis are between $150–$300. Since each transgenic event must be evaluated individually, the cost of rigorously testing an unknown food sample for all the possible current transgenic events is cost prohibitive.

These cost constraints make it necessary to screen samples for DNA components that are present in

most GMOs prior to any quantitative analysis. This type of screening analysis can be carried out with a commercially available PCR test kit, such as the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). This kit provides a protocol and reagents for a nested multiplex PCR assay for the detection of DNA from modified organisms containing the 35S promoter. This genetic element is derived from the cauliflower mosaic virus and is found in most transgenic crops. In addition to the 35S promoter, the multiplex PCR reaction also detects sequences for soya (lectin), corn (zein) and an internal positive control. These multiplex PCR products are all easily resolved and detected using the DNA 500 LabChip and the Agilent 2100 bioanalyzer.

Experimental

DNA Extraction

Three DNA extraction protocols were evaluated prior to the PCR analysis. These protocols were the DNeasy Plant Mini Kit from Qiagen N. V. (Venlo, Netherlands), the Wizard Genomic DNA Purification Kit from Promega (Madison, Wisconsin) and a cetyltrimethylammonium bromide (CTAB) precipitation procedure that was developed in-house. Detailed descriptions of the DNeasy and Wizard Genomic kit protocols can be found in the technical manuals that accompany these products. The CTAB protocol is described below.

CTAB Extraction protocol:

1. A 50-mg sample was added to a 1.0-mL aliquot of CTAB extraction buffer (0.055 M CTAB (cetyltrimethylammonium bromide), 0.1 M Tris, 1.4 M NaCl, 0.2 M disodium EDTA pH 8.0), heated at 65 °C for 15 min and then placed on ice.
2. The extract mixture was centrifuged at 15,000 g for 10 min to remove particulates.
3. The supernatant was removed and stored in a new microfuge tube at 4 °C. (Samples with high starch levels, for example corn, were treated with 0.2 µL of α-amylase and incubated at 37 °C for 30 min.)
4. The recovered supernatant was combined with an equal volume of chloroform mixed for 30 s and then centrifuged for 10 min at 15,000 g. The upper (aqueous) phase was transferred to a new tube.
5. Two volumes of the CTAB precipitation solution (0.014 M CTAB, 0.04 M NaCl) were added to aqueous extract and mixed.
6. The mixture was incubated at 25 °C for 30 min and then centrifuged for 10 min at 15,000 g. The supernatant was discarded.
7. The precipitate was redissolved by adding 250 µL of the 1.2 M NaCl and incubating 37 °C for 10 min. The mixture was centrifuged for 5 min at 15,000 g and the supernatant was transferred to a new tube.
8. One volume of isopropanol was added to the mixture and then the mixture was stored at 4 °C for at least 30 min.
9. The solution was centrifuged for 10 min at 15,000 g and then the supernatant was discarded.
10. The pellet was washed with 500 µL of cold 70% ethanol, the mixture was centrifuged for 5 min at 15,000 g, and then the ethanol solution was discarded.
11. After drying the tube, the DNA pellet was redissolved in 25 µL of Tris buffer (0.01 M Tris, 0.001 M EDTA pH 8.0).

The soya reference standards were used in the evaluation of the DNA extraction because soya contains a high level of protein that can be accurately tracked through each step of the extraction procedure. Although the soya protein is not problematic for the PCR assay, it serves as a useful indicator of overall DNA purity. Protein levels in the final DNA extracts were determined by a protein measurement using the Protein 200 Plus LabChip and the Agilent 2100 bioanalyzer. Absorbance measurements were made with the ND-1000 spectrophotometer from NanoDrop Technologies, Inc. (Wilmington, Delaware).

PCR Protocol

Samples used in the GMO analysis consisted of Institute for Reference Materials and Measurements (IRMM) Certified Reference GMO Soy and GMO Corn, as well as commercial corn meal and soya powder. The PCR analysis was carried out using the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). A modified PCR cycling protocol was developed for use with the PTC 200 Peltier Thermal Cycler from MJ Research, Inc. (Waltham, Massachusetts). The PCR protocol is described below.

Multiplex PCR Protocol

1. Dilute the DNA sample to an initial concentration of 5–50 ng/µL. (This corresponds to an optical density, (OD), at 260 nm of 0.1–1.0 for a 1-cm cell.)
2. For the first stage of the multiplex PCR combine the following per reaction:

   17-µL 2X Qiagen Multiplex PCR Master Mix

   27-µL Allin Mix 1

   5-µL Internal control

   1-µL Extracted DNA
3. Vortex the solution.
4. Perform first stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	55
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

5. For the second stage of the Multiplex PCR combine the following per reaction

   24.5-µL 2X Qiagen Multiplex PCR Master Mix

   24.5-µL Allin Mix 2

   1.0-µL Product from first stage PCR
6. Perform second stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	57
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

Analysis of PCR Products

The PCR products were analyzed using the DNA 500 LabChip and the Agilent 2100 bioanalyzer. For comparison, the same PCR products were also characterized by gel electrophoresis in a 4% NuSieve 3:1 Plus Gel. The electrophoretic separation conditions were 150 V for 60 min in a 1X Tris-borate-EDTA (TBE) buffer.

Results

DNA Extraction

DNA extraction protocols were evaluated for both yield and purity of DNA. Protein levels in the DNA extracts were determined using the Protein 200 Plus LabChip [3]. The results of this comparison are summarized in Table 1.

Table 1. Summary of DNA Extraction

Method	Yield (%)	DNA/Protein w/w	A_{260}/A_{280}
CTAB	0.008	2.7	1.82
DNeasy	0.004	2.5	1.89
Wizard Genomic	0.008	0.3	1.62

The CTAB and Wizard Genomic protocols showed the highest levels of DNA recovery with final DNA yields of 0.008% of the initial sample weight. The DNA/protein ratio of 0.3 found in the Wizard DNA extract indicated that this extract still contained a high level of protein. The corresponding ratio for CTAB was 2.7, indicating that the protein content of the CTAB extract was only 1/9 of the Wizard protocol.

The A_{260}/A_{280} ratio is also a useful indicator of DNA purity, with high purity DNA having an absorbance ratio of 1.8–1.9. A comparison of the A_{260}/A_{280} ratios for the CTAB and Wizard extracts, 1.82 and 1.62 respectively, confirmed the higher DNA purity in the CTAB process. The DNeasy extraction process produced DNA of comparable quality to the CTAB process, but at a significantly lower yield. Since the CTAB extraction protocol gave the highest yield of high purity DNA, this protocol was used to prepare the DNA extracts used in the PCR analysis.

Analysis of PCR Products

The Biosmart Allin 1.0 GMO Screening System is a multiplex PCR kit that is capable of generating four PCR products. These PCR products include the following:

Base pairs (bp)	Product
118	Soy lectin gene
150	35S Promoter
217	Internal control (corn zein sequence added to the PCR mix)
278	Corn zein gene

All four of these products can be found in either the positive or the negative control reactions using the Biosmart GMO Screening System. The DNA 500 LabChip has ample separation to resolve all of these PCR products. This resolution is illustrated in Figure 1, which shows a composite of the bioanalyzer electropherograms for the positive and negative controls.

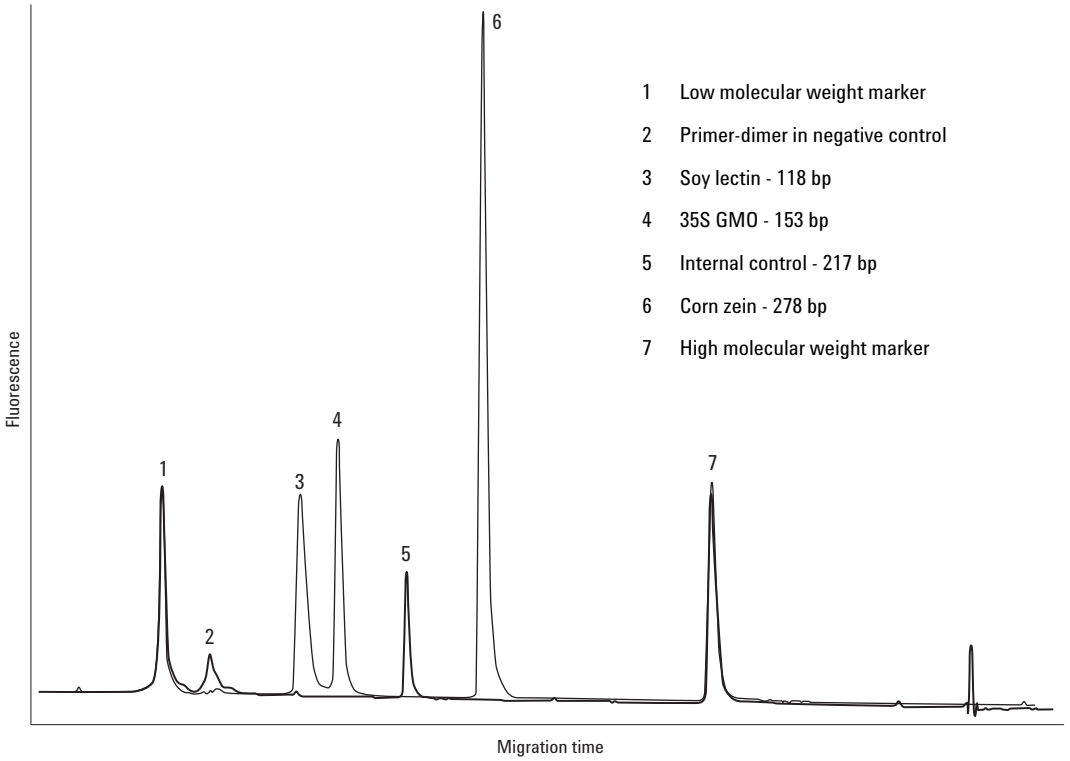

Figure 1. Composite of bioanalyzer electropherograms for positive and negative controls.

The PCR analysis was carried out on corn and soya sample sets. The corn sample set consisted of IRMM Certified Reference MON810™ corn standards at the following levels: 0% MON810, 0.1% MON810, 0.5% MON810, 1.0% MON810, 2.0% MON810, and 5.0% MON810, and commercial corn meal. The soya sample set was made of IRMM Certified Reference Roundup Ready® soya samples at the following levels: 0% Roundup Ready, 0.1% Roundup Ready, 0.5% Roundup Ready, 1.0% Roundup Ready, 2.0% Roundup Ready, and 5.0% Roundup Ready and commercial soya powder. Both sample sets contained a positive control with 0.5% each Bt176 corn and Roundup Ready soya, and deionized water as a negative control.

Figure 2 shows the electrophoretic gel separation and a gel-like bioanalyzer image for all of the samples listed above. The PCR products for the corn samples are shown in Figures 2A and 2C. Figures 2B and 2D show the soya results.

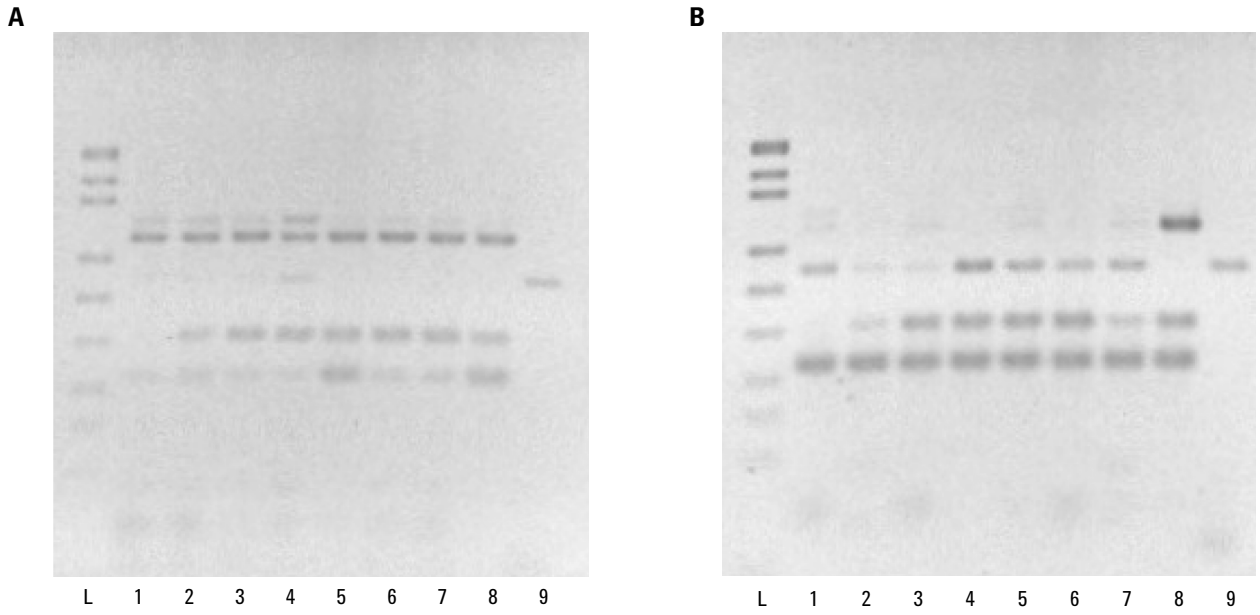

Figure 2A and 2B. 4% NuSieve 3:1 Plus Gel, 150 V for 60 min.

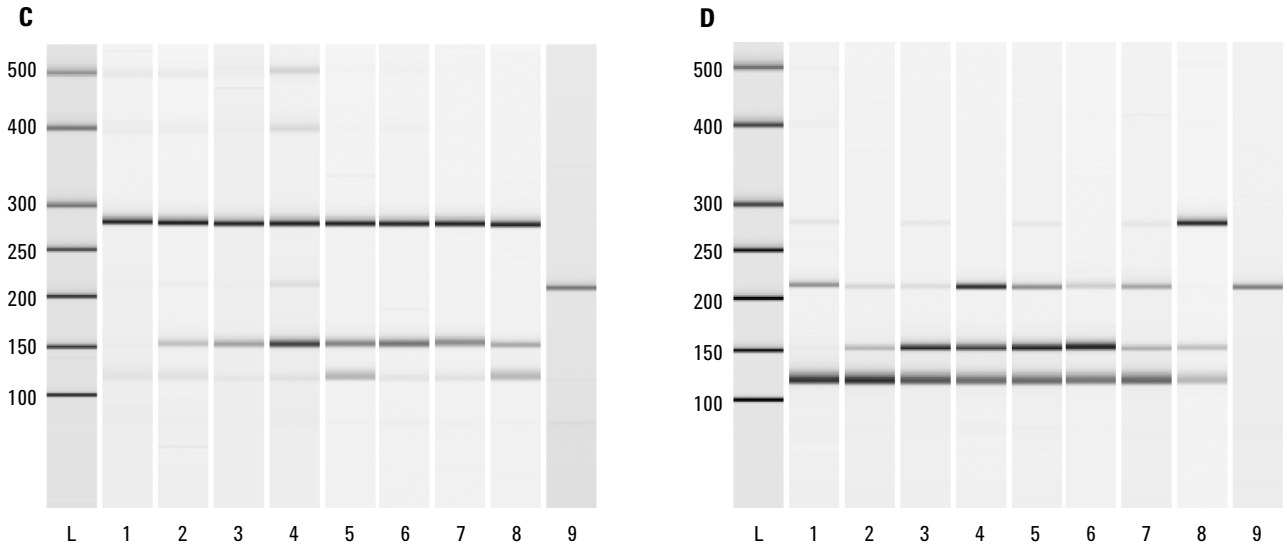

Figure 2C and 2D. Gel-like bioanalyzer image.

Figure 2. 2A (gel) and 2C (bioanalyzer)
L) Molecular weight ladder: 501, 489, 404, 353, 242, 190, 147, 110, 89, 67, 34, 34, and 26 bps **1**) 0% MON810 corn, **2**) 0.1% MON810 corn, **3**) 0.5% MON810 corn, **4**) 1.0% MON810 corn, **5**) 2.0% MON810 corn, **6**) 5.0% MON810 corn, **7**) Commercial corn meal, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

2B (gel) and 2D (bioanalyzer)
L) Molecular weight ladder, **1**) 0% Roundup Ready soya, **2**) 0.1% Roundup Ready soya, **3**) 0.5% Roundup Ready soya, **4**) 1.0% Roundup Ready soya, **5**) 2.0% Roundup Ready soya, **6**) 5.0% Roundup Ready soya, **7**) Commercial soya powder, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

Comparison of Gel and Bioanalyzer Response

The bioanalyzer DNA 500 LabChip clearly shows superior resolution and uniformity of band location compared to the 4% NuSieve gels. The enhanced reproducibility of band location in the gel-like bioanalyzer image is readily apparent in a visual comparison of Figures 2C and 2D to Figures 2A and 2B. Both the 4% gel and the bioanalyzer have sufficient sensitivity to visualize the 35S PCR product at the minimum corn and soy reference standard levels of 0.1% GMO.

A comparison of the initial 35S level in the sample to the amount of 35S PCR product shows that the 35S GMO PCR band increases as the GMO content increases. The PCR response in both corn and soy samples appears to saturate before the maximum GMO standard level of 5% is reached. This effect can be clearly demonstrated using the bioanalyzer's ability to measure PCR product concentrations. Tables 2 and 3 show the concentrations of the corn, soy, and 35S PCR products. For the corn samples, the PCR product response saturates at 1.0% GMO. In the soy samples, the saturation occurs at 0.5% GMO.

Table 2. GMO Corn Response - Corn and 35S PCR Products

Sample	278 bp-Corn amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ corn amplicons
0% MON810 Corn	2.5	0.03	0.01
0.1% MON810 Corn	3.4	1.2	0.4
0.5% MON810 Corn	6.2	3.8	0.6
1.0% MON810 Corn	3.5	4.3	1.2
2.0% MON810 Corn	5.2	4.4	0.9
5.0% MON810 Corn	4.7	4.7	1.0
Corn meal	6	5.2	0.9

Table 3. GMO Soya Response - Soya and 35S PCR Products

Sample	118 bp-Soya amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ soya amplicons
0% Roundup Ready soy	5.1	0.1	0.02
0.1% Roundup Ready soy	5.4	0.8	0.15
0.5% Roundup Ready soy	4.9	3.4	0.7
1.0% Roundup Ready soy	5.3	4.0	0.8
2.0% Roundup Ready soy	5.1	4.4	0.9
5.0% Roundup Ready soy	4.8	5.0	1.0
Soya powder	4	0.9	0.2
Positive control	4.8	2.2	0.5

Although concentrations of PCR products can be determined quite accurately, some care must be exercised in the interpretation of these quantitative results. Under conditions where a correlation can be seen between the GMO content and the concentration of GMO amplicon, for example, GMO <0.5%, a rough estimate of GMO concentration can be made. However, such an estimate is only reliable if the same PCR master mix and thermocycler are used for all the amplification reactions. In addition, sample and calibration standard matrices must also be highly similar.

For the Biosmart GMO Screening System, quantitative conclusions cannot be made at concentrations >0.5% GMO because the PCR response is saturated. Once the PCR product concentrations have reached this level, small differences in amplicon concentrations are not useful in making quantitative estimates. An example of this can be seen in Table 3. The 0.5% GMO soy reference has a 35S/soy amplicon ratio of 0.7. In the positive control, where the sample has 0.5% GMO soy and 0.5% GMO corn, the corresponding ratio is only 0.5. Since the positive control contains at least twice as much 35S GMO marker as the 0.5% soy reference, this ratio would be expected to be greater than 0.7. It is difficult to explain why the ratio is lower in the positive control. This behavior could be the result of the complex reaction kinetics in a multiplex nested PCR assay or may simply reflect the imprecision in endpoint PCR amplicon concentrations.

PCR Product Composition

The internal control in the Biosmart Allin 1.0 GMO Screening System is a 217 bp corn sequence that uses the same primer sequences as the corn PCR product. According to the manufacturer, when high levels of corn DNA are present, competition for primer may result in the loss of the internal control PCR product. An examination of the corn PCR products in Figure 2C shows this effect in that the 217 bp is either absent or visible only at trace levels. However, in the soy PCR assay in Figure 2D, the 217 bp fragment can easily be seen in all the samples except the positive control that contains corn DNA.

In the corn sample set, a weak band can be seen at around 120 bp. This suggests that during the DNA isolation step, trace amounts of soya DNA were introduced into the corn samples. Likewise, the presence of a band at 280 bp in some of the soy samples indicates low levels of corn DNA were present in several of the soy samples. It is not surprising that trace levels of cross contamination should be apparent in a nested PCR assay. Since all of the samples undergo a net 80 cycles of amplification, even a few copies of soy or corn DNA will be sufficient to produce a detectable PCR product.

In PCR assays using a large number of amplification cycles, it is not uncommon for amplicons of similar sequence to cross hybridize. In the case of the Biosmart Allin 1.0 GMO Screening System, both the corn PCR product and the internal control share a region of common DNA sequence. When these two amplicons cross-hybridize, the resulting product has both single-stranded and double-stranded regions. These structures, known as heteroduplexes, have substantially lower mobility than a corresponding double-stranded structure. The relative mobility shift depends on such factors as gel composition, ionic strength, and gel temperature [4]. In Figure 2, PCR products that are larger than the 278 bp corn amplicon are observed. These bands occur at about 320 bps in the 4% gel and at 400 and 500 bps in the bioanalyzer electropherogram. Cross hybridization of the corn and the internal control amplicons is probably responsible for these products.

Conclusion

This application note described the use of the Agilent 2100 bioanalyzer with the Protein 200 Plus and DNA 500 LabChip Kits in the evaluation of sample preparation and the analysis of multiplex PCR products. The Protein 200 Plus was used to determine which DNA extraction procedure was most effective in removing residual protein. The DNA 500 LabChip was used to characterize the Biosmart Allin 1.0 GMO Screening System, a nested multiplex PCR assay for the genetically modified corn and soy beans. Resolution and sensitivity in these assays was sufficient to identify all of the targeted multiplex PCR amplicons and to differentiate these targets from PCR artifacts. Sensitivity of the assay was sufficient to detect GMO content even at the minimum GMO standard level of 0.1% in both corn and soy reference standards.

References

1. S. Vollenhofer, K. Burg, J. Schmidt and H. Hroath, “Genetically Modified Organisms in Food - Screening and Specific Detection by Polymerase Chain Reaction” (1999) *J. Agric. Food Chem.* **47**, 5038-5043.
2. S. Garret, O. Arun and J. Dooley, “Analysis of Genetically Modified Soya Using the Agilent 2100 bioanalyzer”, Agilent Technologies, publication 5988-4070EN www.agilent.com/chem.
3. D. Dempsey, and M. Jensen, “Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 bioanalyzer”, Agilent Technologies, publication 5988-9441EN, www.agilent.com/chem.
4. M. A. Jensen, and N. Straus, “Effect of PCR Conditions on the Formation of Heteroduplex and Single-stranded DNA Products in the Amplification of Bacterial Ribosomal DNA Spacer Regions”, (1993) *PCR Methods and Applications* **3**, 186.

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Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer

Application

Food Safety

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Abstract

This application note shows how the Agilent 2100 Bioanalyzer was used in polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fragment analysis for fish species identification. A 464bp cytochrome-b target sequence, found in all vertebrate fish, was first amplified and then digested with restriction enzymes. The fragments were resolved on the DNA 500 LabChip®, allowing simple comparison with authentic species profiles due to the accuracy of fragment size determination. Use of the Bioanalyzer offered significant benefits over traditional gel electrophoresis and DNA staining techniques for PCR-RFLP fragment analysis.

Introduction

The diversity of fish products available to consumers has increased significantly in recent years. Products can range from premium grade fish steaks to low cost fish fingers. As fish are caught, processed, and distributed by a global network of operators, there is a need to ensure the authenticity and the origin of fish used in the products.

There is, therefore, a need to have reliable and simple species identification methods to support enforcement and compliance with labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001).

Methods of fish species identification based on morphological characteristics are suited to whole fish; however, fish species identification becomes more problematic once it is processed. Protein profiling is used for fish identification; however, this method requires the analysis of species reference materials alongside the samples and is less reliable when applied to processed food products as the proteins become denatured. DNA based methods offer an alternative approach to species identification as DNA remains detectable in all but the most heavily processed samples.

Direct sequencing is the most definitive method of identification; however, it cannot easily be applied to samples suspected or known to contain more than one species. Alternative techniques, using polymerase chain reaction (PCR), were developed to identify fish species based on DNA fingerprint patterns. Methods used include RAPDs (random amplified polymorphic DNA), SSCP (single strand conformation polymorphism) and PCR-RFLPs.

A PCR-RFLP technique, which involved digesting an amplified 464bp region of the cytochrome b gene with restriction enzymes to generate DNA profiles, was previously developed for the identification of salmon species [1, 2].

The aim of this work was to improve the method for identification of salmon and other species by replacing conventional gel electrophoresis and staining steps with the Agilent 2100 Bioanalyzer. The generation of species-specific PCR-RFLP profiles on the 2100 Bioanalyzer combined with accurate sizing and quantification of individual DNA fragments, offered significant advantage over gel-based approaches in terms of ease-of-use, speed, and accuracy of identification.

Materials and Methods

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich and were of molecular biology grade or equivalent. PCR primers were supplied by MWG-Biotech UK Ltd. PCR-RFLP profiles were generated using a DNA500 LabChip and the Agilent 2100 Bioanalyzer. AmpliTaq® Gold DNA polymerase from Applied Biosystems was used in all PCR reactions. Restriction enzymes were obtained from New England Biolabs and used per the manufacturer's instructions.

Fish Samples

Commercially important salmon and white fish species samples were obtained from appropriate fishery research laboratories in the UK, Canada, Alaska, New Zealand, and Japan. Five individuals were used to minimize the effects of polymorphic variation within the population. Additional samples of each fish species were obtained from local UK fishmongers and retailers.

DNA Extraction

DNA extraction was performed using a modification of the CTAB method. Samples (2 g wet weight) were suspended in 5 mL of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100-mM Tris-HCl, 20-mM EDTA, 1.4-M NaCl, pH 8.0) and 40 µL of Proteinase K solution (20 mg/mL) was added. Samples were mixed thoroughly and then incubated overnight at 60 °C. After incubation, 1 mL of supernatant was transferred to a 2.0-mL Eppendorf tube, cooled to room temperature (RT), and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform was added. The solution was vortexed and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was transferred to a clean 1.5-mL Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at RT for 30 minutes. DNA was pelleted by centrifugation at 16,000g for 15 minutes, washed in 70% ethanol and air dried for 30 minutes at RT. The DNA pellet was resuspended in 100 µL of sterile distilled water (SDW) and purified using Promega's Wizard® Purification Resin per the manufacturer's protocol. DNA extracts were recovered in 50 µL of 1×TE (10-mM Tris-HCl, pH 7.4, 1-mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µL) were determined using a GeneQuant pro DNA calculator (Pharmacia).

DNA Amplification

PCR products (464bp target from the cytochrome b gene) were produced by amplification of DNA extracts (50 ng) in 20-µL reactions containing 1× AmpliTaq Gold PCR buffer (Applied Biosystems), 300 nM of each primer (L14735: 5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3' and H15149: 5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3'), 200-nM dNTPs, 5-mM $MgCl_2$ and 0.05-U/µL of AmpliTaq Gold (Applied Biosystems). Amplification profiles (94 °C for 5 minutes [denaturation]; 40 cycles of: 94 °C for 40 s, 50 °C for 80 s, 72 °C for 80 s [amplification]; 72 °C for 7 minutes [final extension] were applied using a PE9600 PCR machine (Applied Biosystems). Unpurified PCR products (1 µL) were applied to the 2100 Bioanalyzer to confirm amplification.

PCR-RFLP Profiling

Unpurified PCR product (2.5 µL) was digested for 3 or more hours with two to five units of enzyme in a total volume of 5 µL. Reactions were terminated by incubation at 65 °C for 10 minutes. Digested PCR products (5 µL) were mixed with 5 µL of 20-mM EDTA, to achieve a final concentration of 10-mM EDTA, prior to loading on to DNA500 LabChips. Aliquots (1 µL) of the reaction mix were analyzed on the 2100 Bioanalyzer, per manufacturers' instructions.

Results

Evaluation of PCR-RFLP Profiles Generated on the 2100 Bioanalyzer for Species Identification

An initial evaluation of the PCR-RFLP method was performed using salmonid species.

Following cleavage of the amplified DNA fragment with restriction enzymes, species-specific PCR-RFLP patterns were resolved on the 2100 Bioanalyzer. An example of a PCR-RFLP pattern is given in Figure 1, which shows PCR-RFLP profiles for salmon and trout samples generated with enzymes DdeI and HaeIII.

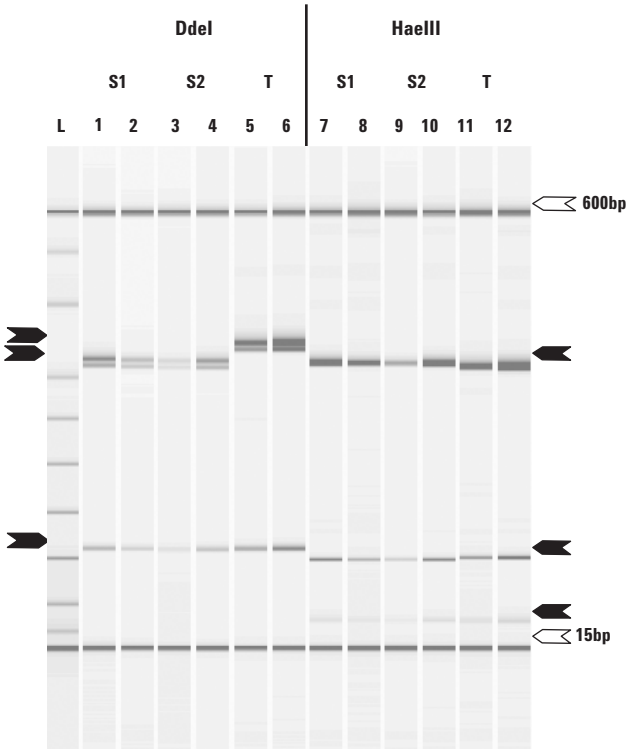

Figure 1. PCR-RFLP patterns obtained from salmon and trout with enzymes DdeI and HaeIII. PCR-RFLP patterns obtained when amplified DNA from two salmon (S1, S2) and one trout (T) samples were digested with DdeI (lanes 1–6) or HaeIII (lanes 7–12). A 15bp–600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).

Observed and expected fragment sizes for a selection of five enzymes and four salmonids are shown in Table 1. As can be seen, patterns were similar to those reported previously [1, 2].

Expected DNA fragments of greater than about 25bp were readily detected; however, some smaller fragments were not detected because they could not be distinguished from the lower 15bp size marker or were outside the detection range (25bp–500bp) of the LabChip. Small DNA fragments (25bp–100bp), which were not reported previously, were observed in some digests [2]. This highlights the improved band resolution of this method in comparison to gel electrophoretic methods. This improved resolution is also highlighted by profiles generated with DdeI, where all four species have an extra fragment that is about 9bp smaller than the expected larger fragment. This is due to an extra DdeI site situated in primer H15149

Table 1 shows that *O. gorbuscha* and *O. mykiss* profiles generated with NlaIII have only two fragments when three are expected. This is believed to be due to the comigration of the two larger fragments. An analysis of the sequence of the *O. mykiss* 464bp amplicon indicated that cleavage of the amplicon with NlaIII should produce fragments of 192bp, 180bp and 91bp. These respectively equate to the 210bp, 190bp and 100bp fragments reported by Russell *et al.* (2000) and appear in Table 1. From the sequence data there is

Table 1. Expected and Observed PCR-RFLP Fragment Sizes Obtained with Five Restriction Enzymes and Four Salmonid Species

		Expected* (E) and observed (O) fragment sizes for each enzyme (bp)				
Species		**DdeI****	**Bsp1286I**	**HaeIII**	**NlaIII**	**Sau3AI**
O. nerka						
(Red salmon)	E	360, 130	300, 200	350, 130	310, 180	390, 120
	O	353, 346, 114	289, 172	320, 102, 35 or 421	272, 160	340, 115
O. gorbuscha						
(Pink salmon)	E	360, 130	U/C***	U/C	210, 190, 100	390, 120
	O	349, 343, 112	464	421	181, 92	338, 115
S. salar						
(Atlantic salmon)	E	350, 130	300, 200	350, 130	U/C	410, 110
	O	321, 312, 110	287, 172	318, 98, 35	438	370, 88
O. mykiss						
(Rainbow trout)	E	360, 130	300, 200	350, 130	210, 190, 100	U/C
	O	348, 339, 111	279, 174	313, 100, 33	185, 92	451

*Sizes as reported by Russell *et al.* (2000).

**Extra fragments in observed DdeI profiles are due to restriction site introduced by primer H15149.

***U/C Uncut with enzyme.

no evidence that the smaller 180bp fragment contains a higher proportion of heavier A or G bases or the larger 192bp fragment a higher proportion of lighter C or T bases, which could cause their respective molecular weights to converge. The calculated molecular weight difference (3277 Daltons) between the two fragments is equivalent to the difference in the number of bases. This makes it unlikely that comigration is due to molecular weight similarities between the two fragments. The comigration of these two fragments as a single fragment is consistently observed and does not detract from the identification of these species.

Overall, the profiles generated by the 2100 Bioanalyzer matched those expected or previously reported [1, 2], which supports the use of this approach for the identification of fish species. Further work was performed using Atlantic salmon and trout and a smaller number of enzymes to confirm the application of this approach.

Experimental Repeatability

In order to determine the experimental repeatability (LabChip-to-LabChip variability) of the complete assay, duplicate PCRs were produced from two salmon and one trout sample. Amplified fragments were cleaved with DdeI and digests stored at 4 °C until required. PCR-RFLP patterns were separated on four occasions using different DNA500 LabChips primed with two different batches of gel matrix, A and B. Two LabChips were run using a freshly prepared gel matrix (matrix A) while a third LabChip was run when gel matrix A was 1-week old. The fourth LabChip was run on the same date as the third LabChip but using a second, fresh batch of gel matrix (matrix B). Overall variation (encompassing variation due to LabChip-to-LabChip, PCR and gel matrix) appears in Table 2, which shows the results of analysis with the four LabChips following digestion with enzyme DdeI. Results are the mean fragment sizes observed on each LabChip from two PCR replicates of each species. Absolute fragment size variations within a single LabChip, that is, for PCR replicates of the same sample or between the two salmon samples, were less than 2bp and were only observed between the larger (>300bp) fragments. The overall absolute size variation for each fragment, which included variation due to different LabChips, PCRs and gel matrices, was slightly greater; the biggest variation was 6bp for the 320bp fragment in salmon (321bp to 327bp).

Fish Species Identification

Using sequence data generated from 10 different white fish species, theoretical PCR-RFLP profiles were generated from a range of restriction enzymes. Closer examination of these theoretical profiles indicated that only three enzymes would be needed to differentiate all the white fish species. Experimental profiles were generated to confirm that species identification was possible using just these three enzymes. Results of this analysis are shown in Figure 2, which shows that 9 of the 10 species could be identified based on profiles generated with only one enzyme. The remaining two species were differentiated using the other two enzymes.

Table 2. PCR-RFLP Fragment Sizes Obtained Following Separation of DNA Cleaved with DdeI on Four Different DNA500 LabChips

	Observed fragment sizes on each LabChip (bp)					
Expected band size (bp)	LabChip 1 (Fresh matrix A)	LabChip 2 (Fresh matrix A)	LabChip 3 (Week old matrix A)	LabChip 4 (Fresh matrix B)	Mean	%RSD
Salmon analysis*						
117	111	111	110	111	111	0.34
312	314	316	314	317	315	0.43
320	323	325	323	326	324	0.51
Trout analysis						
116	111	111	110	111	111	0.45
339	338	341	338	343	340	0.72
348	347	349	347	352	349	0.61

*An expected 27bp fragment from salmon is not detected by the 2100 Bioanalyzer.

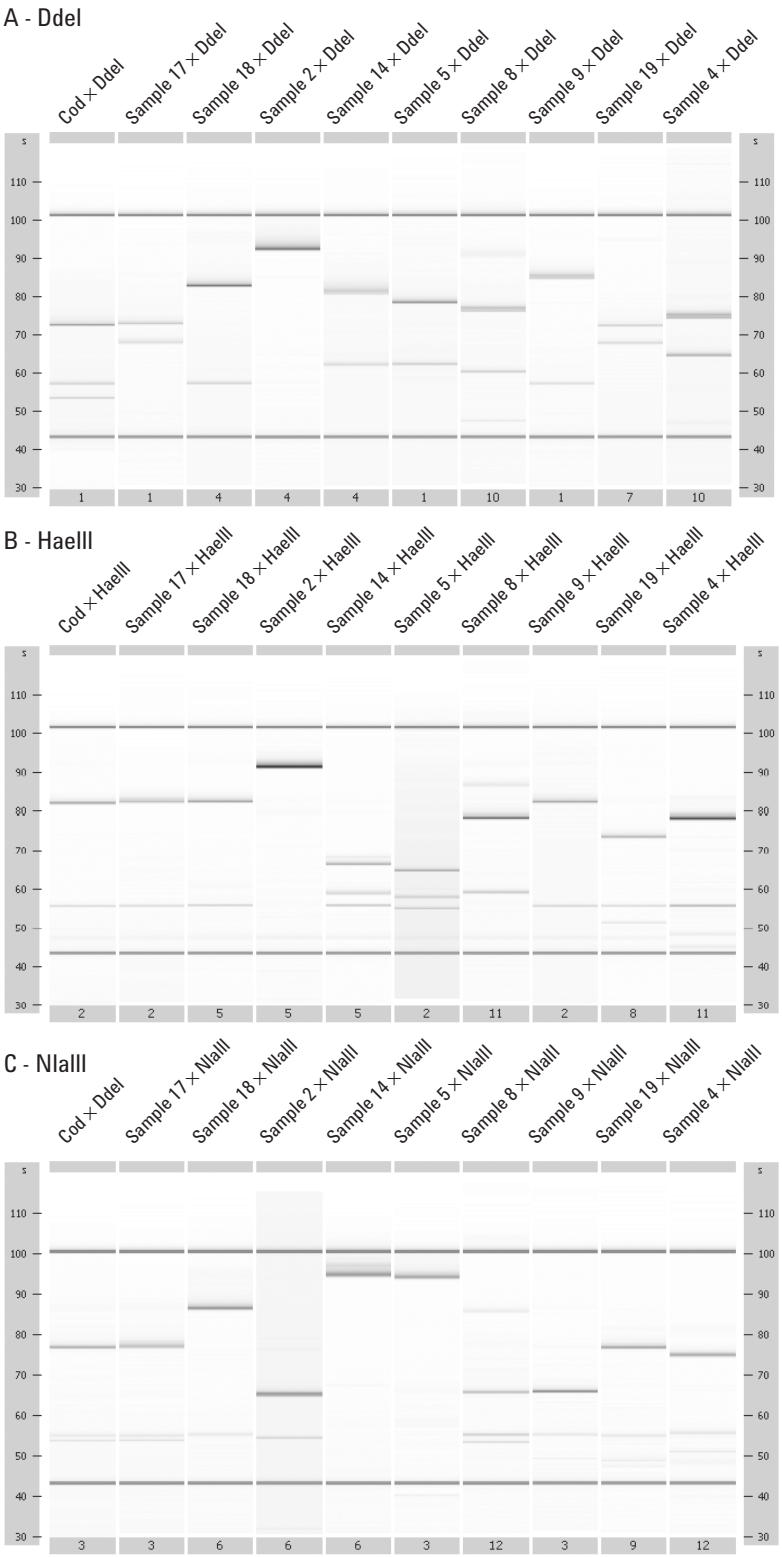

Figure 2. PCR-RFLP profiles from the 10 white fish species used in this study. Profiles were generated using enzymes DdeI (A), HaeIII (B) or NlaIII (C). Each sample number indicates a different fish species.

Analysis of a further nine salmon species was performed using these three enzymes only (data not shown). Again it was found that the salmonids could be differentiated using the three enzymes. A list of all the species studied is found in Table 3.

Table 3. Fish Species that Could be Differentiated Using PCR-RFLP with Enzymes Dde I, Hae III and NlaIII

Common name (UK)	Latin name
Atlantic cod	*Gadus morhua*
Pacific cod	*Gadus macrocephalus*
Coley (Saithe)	*Pollachius virens*
Haddock	*Melanogrammus aeglefinus*
European hake	*Merluccius merluccius*
South African hake	*Merluccius paradoxus*
European plaice	*Pleuronectes platessa*
Whiting	*Merlangus merlangus*
Alaskan(Walleye) Pollock	*Theragra chalcogramma*
Hoki	*Macruronus novaezelandiae*
Atlantic salmon	*Salmo salar*
Red / Sockeye salmon	*Oncorhynchus nerka*
Pink / Humpback salmon	*Oncorhynchus gorbuscha*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho / Silver salmon	*Oncorhynchus kisutch*
Keta / Chum salmon	*Oncorhynchus keta*
Cut-throat trout	*Oncorhynchus clarki clarki*
Dolly Varden	*Salvelinus malma malma*
Cherry salmon	*Oncorhynchus masou masou*

Discussion

To identify species present in a sample when no prior knowledge of the sample is available requires a universally applicable method. PCR-RFLP profiling of a common region of the vertebrate cytochrome b gene, which is present in all fish species, enabling comparison with profiles in a database is one such universal approach.

The conventional PCR-RFLP fragment analysis involves gel electrophoresis, on large (over 30 cm^2), thin (<2 mm) acrylamide gels, to resolve the PCR-RFLP patterns. This makes handling and staining difficult and requires the use of large equipment and volumes of solution. All this makes these methods potentially hazardous and time consuming and can sometimes produce variable results. This type of detection is, therefore, not suited to use in enforcement and quality control laboratories where a rapid, robust detection method is required.

As an alternative, the 2100 Bioanalyzer incorporates conventional capillary electrophoresis (CE) technology into an easy-to-use chip-based format, which enables accurate sizing and quantification of individual DNA fragments. Coupled with the small (2 cm^2) size of the LabChip, this gives the system a significant advantage over conventional gel-based approaches in terms of ease-of-use, speed, and safety. This makes the 2100 Bioanalyzer ideally suited to the analysis of multiple small DNA fragments such as those found in PCR-RFLP profiles.

Using the 2100 Bioanalyzer it was possible to generate PCR-RFLP profiles that resembled those previously published for salmon species. However, generating PCR-RFLPs on the 2100 Bioanalyzer produced profiles with improved fragment resolution and detection, especially of smaller fragments that were not detected using conventional gel electrophoresis. PCR-RFLP profiles generated on the 2100 Bioanalyzer were also more consistently produced compared to gel electrophoresis.

Consequently, profiles based on three enzymes DdeI, NlaIII and HaeIII were developed for 19 commercially important species. Further studies should increase the range of species in the database, making it an important tool for the study of the authenticity of fish products.

Complete details of the development of the assays, application to fish species identification in products, and an interlaboratory study were recently published [3, 4, 5].

Acknowledgements

This work formed part of the UK's Food Standards Agency project Q01069 and the authors wish to acknowledge the FSA for their financial support.

References

1. G. L. Hold, V. J. Russell, S. E. Pryde, H. Rehbein, J. Quinteiro, M. Rey-Mendez, *et al.*, (2001), Validation of a PCR-RFLP based method for the identification of salmon species in food products. *European Food Research and Technology,* **212**, 385–389.
2. V. J. Russell, G. L. Hold, S. E. Pryde, H. Rehbein, J. Quinteiro, M. Rey-Mendez, *et al.*, (2000). Use of restriction fragment length polymorphism to distinguish between salmon species. *Journal of Agricultural and Food Chemistry*, **48** (6), 2184–2188.
3. J. J. Dooley, H. D. Sage, H. M. Brown, and S. D. Garrett, Improved fish species identification by use of lab-on-a-chip technology. (2005) *Food Control* **16** (7), 601–607.
4. J. J. Dooley, M. A. Clark, H. D. Sage, H. M. Brown, and S. D. Garrett, (2005). Fish species identification using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products and an Inter-laboratory study. *Journal of Agricultural and Food Chemistry*, **53** (**9**), 3348–3357.
5. J. J. Dooley, M. A. Clark, H. D. Sage, H. M. Brown, and S. D. Garrett, (2004) Application of a chip-based capillary electrophoresis system to enable simple PCR-RFLP identification of fish species. FSA Final Report, Q01069

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Agilent 2100 bioanalyzer

Application compendium

Agilent Technologies

Agilent 2100 bioanalyzer

Application compendium

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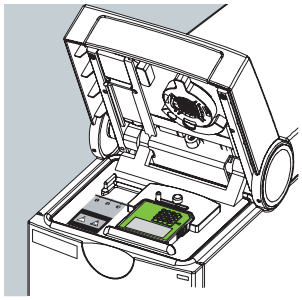

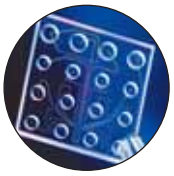

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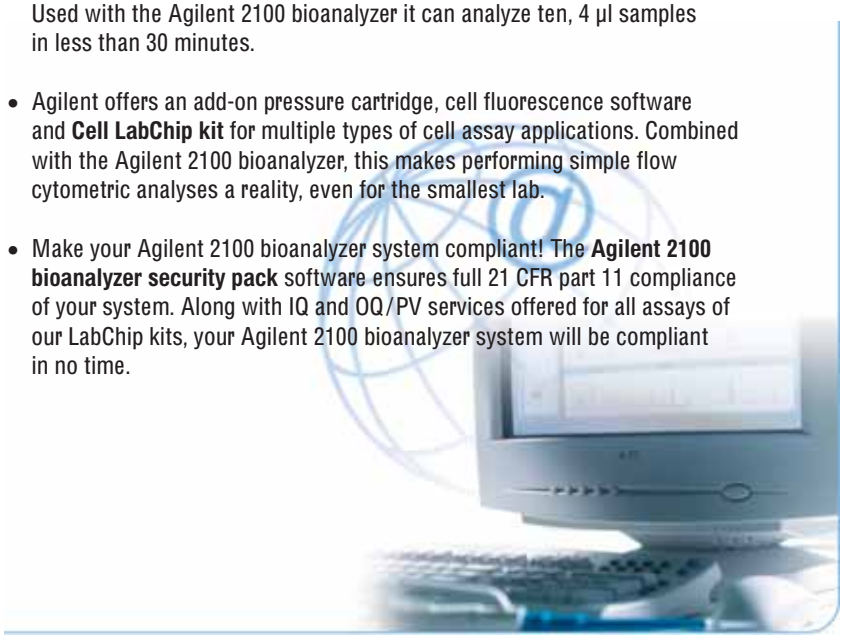

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Cell surface antibody staining - CD3 in T-cell leukemia		●			●	●	
CD3 expression in T-cell leukemia via on-chip staining		●			●	●	
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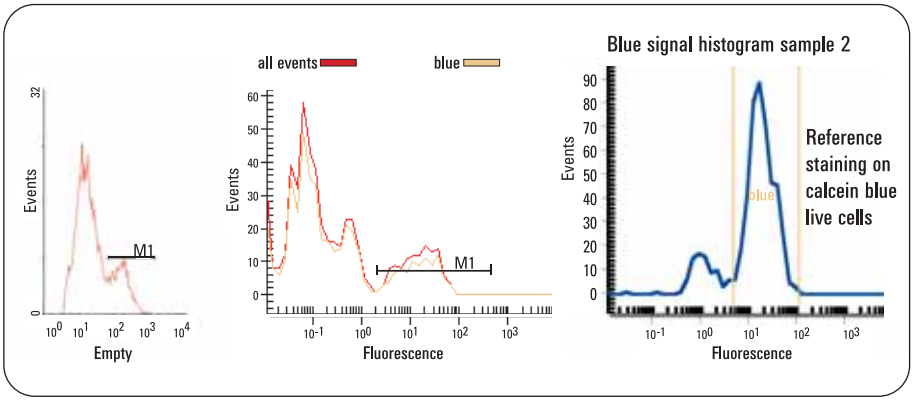
Protein expression monitoring

Cell surface antibody staining - CD4 in CCRF CEM T-cells

Flow cytometer (10,000 Events) Agilent 2100 bioanalyzer (500 Events)



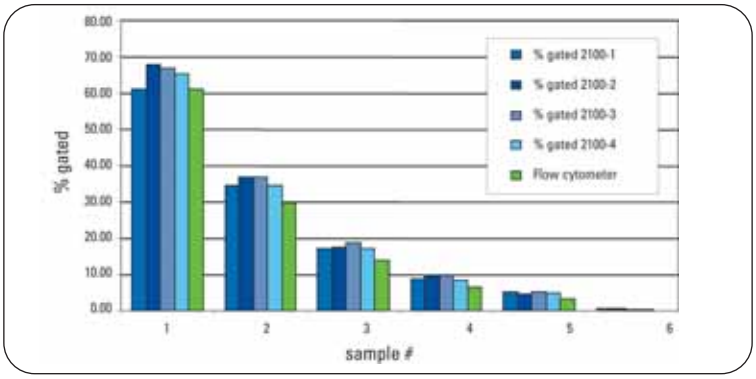
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: CCRF-CEM cells were stained with hCD4-APC labeled antibodies and calcein live dye. 65% of all CCRF-CEM live cells (yellow curve) are expressing CD4 protein which is good in comparison to conventional flow cytometer results.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Cell surface antibody staining - CD3 in T-cell leukemia

Averaged data per instrument



Mean % CD3+ cells				
2100-1	2100-2	2100-3	2100-4	Flow cyt.
60.9	67.8	66.6	65.0	60.9
34.4	36.7	36.7	34.3	29.8
17.3	17.6	18.7	17.2	13.8
8.9	9.4	9.9	8.3	6.5
5.1	4.4	5.3	4.9	3.2
0.8	0.6	0.3	0.3	0.0

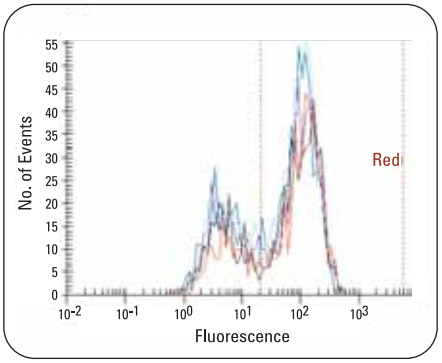
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat (T-cell leukemia) cells were stained with calcein alone or with calcein and APC-labeled anti-CD3 antibody. To mimic different subpopulation sizes, mixtures of both populations were prepared at various ratios.
Samples were analyzed with 4 Agilent 2100 bioanalyzer instruments on 5 chips and compared to a flow cytometer reference instrument. Interestingly, small subpopulations (like 10 - 20%) could be analyzed with good accuracy and reproducibility.

Corresponding application note: 5988-4322EN

Protein expression monitoring

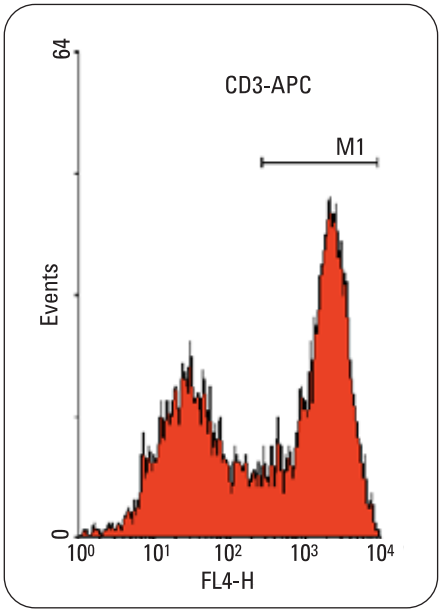
CD3 expression in T-cell leukemia via on-chip staining

A. On-chip Agilent 2100 bioanalyzer



Sample	% of Gated	Sample	% of Gated
1	-	4	64.6
2	66.9	5	66.7
3	67.2	6	72.0

B. Conventional flow cytometry



Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat cells were stained on-chip with anti hCD3-APC prediluted 1:5.5 in cell buffer and Calcein (1:50 in cell buffer). After an incubation time of 25 minutes in the chip, samples were measured in the Agilent 2100 bioanalyzer. The faster and easier on-chip staining procedure has the advantage here of reducing cell consumption 17 fold and antibody reagent costs 80 fold.

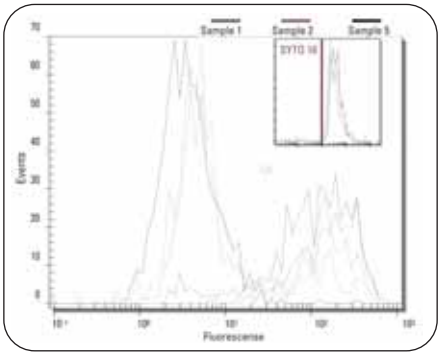
A) Overlay of representative histograms of calcein and antibody treated cells.
B) Comparison between on-chip staining data and data obtained by measuring cells stained by conventional staining on a flow cytometer.

Corresponding application note: 5988-7111EN

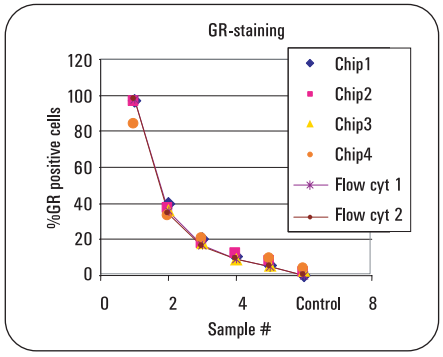
Protein expression monitoring

Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes

Chip histogram overlay from 700 cells/sample



Correlation of chip vs. flow cytometer results

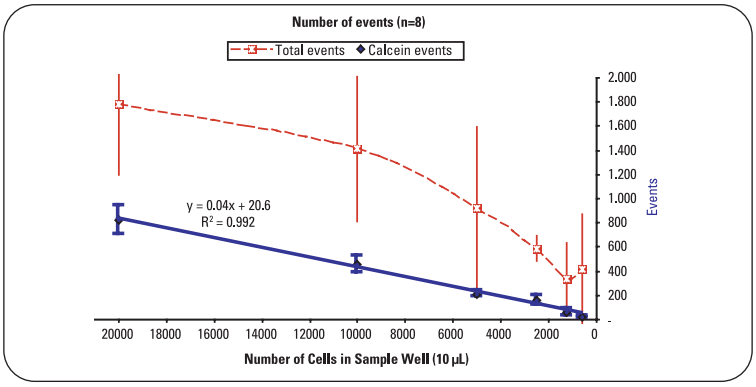


Kit: Cell fluorescence kit
Assay: Generic assay
Application: H4 hepatocytes cells were stained with SYTO16 DNA dye alone or with SYTO16 and GR primary antibody. After washing, both cell preparations were stained with APC-labeled secondary antibody. Mixtures of both populations were prepared at various ratios.
The insert in the left picture shows the overlay of all six cell samples in the blue reference color. The black histogram represents data from the control sample, no GR detected.
All other 5 samples have significant staining above marked fluorescence intensity in the red.
Good chip to chip reproducibility and comparison to flow cytometer is demonstrated.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Analyzing a limited number of cells



Cells	Live-CD3+	STD(n=4)
20,000	83.7%	3.5%
10,000	85.6%	4.1%
5,000	87.7%	4.2%
2,500	84.0%	3.0%
1,250	89.8%	6.5%
625	90.0%	9.3%

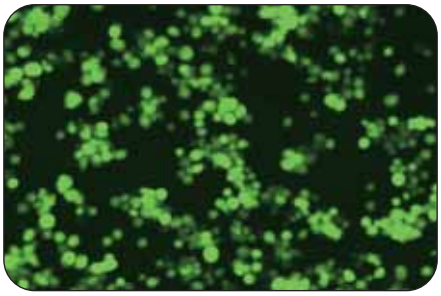
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The direct comparison of different input numbers of cells (down to 625 cells in 10 µl) for the on-chip staining protocol reveals that even with a much lower number than the recommended 20000 cells/10 µl for the standard protocol reliable and meaningful results can be achieved with good reproducibility. The data shown were generated with CD3-positive Jurkat cells stained with an anti-CD3 antibody for the CD3 protein and counterstained with the live cell stain Calcein AM. Similar results were obtained with primary human dermal fibroblasts (PHDF) indicating the usefulness of this method for scarce specimen. The lack of sensitivity, automation and convenient quantitation found with other methods can be circumvented easily by using the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0746EN

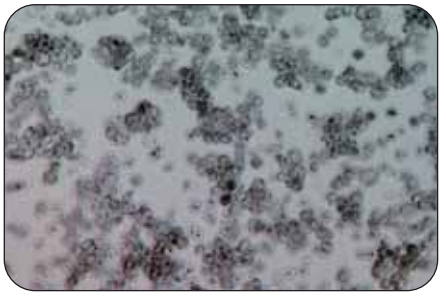
Protein expression monitoring

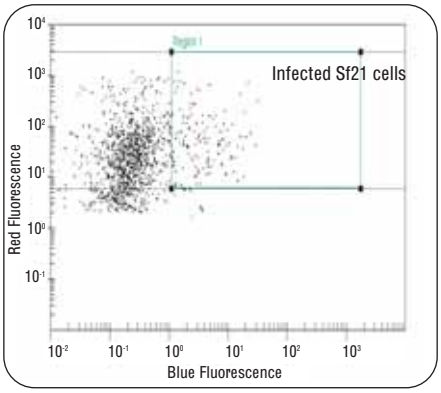
Baculovirus titre determination

Fluorescent Light



Transmitted Light





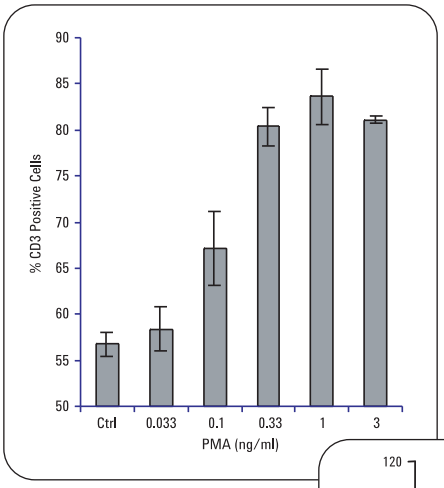
Kit: Cell fluorescence kit
Assay: GFP Assay
Application: A fast and convenient method exists for the calculation of baculovirus titre for expression systems facilitating insect cells. Using GFP-linked co-expression plasmids, the Agilent 2100 bioanalyzer and the flow cytometry set allows the calculation of the viral titre for six samples in approximately 90 minutes. It is superior to traditional plaque assays in terms of labor time, automation and user-to-user variability.

Corresponding application note: 5989-1644EN

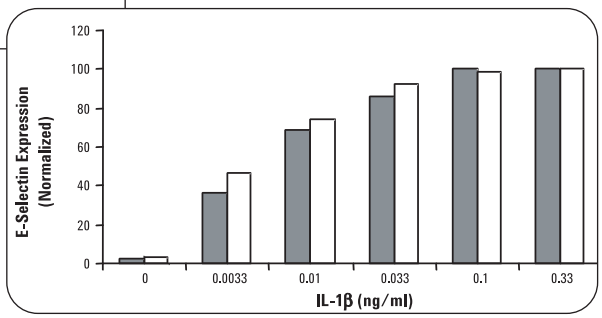
Protein expression monitoring

Upregulated gene expression in primary cells

A



B



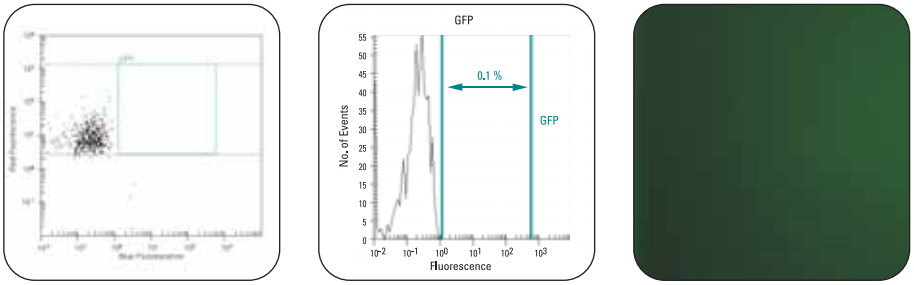
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Flow cytometric analysis of primary cells can present a challenge for researchers due to limited availability and life span of primary cells. A dose-respondent upregulation of protein expression in primary cells using only a minimum number of cells in a fast on-chip-staining approach is shown here. Activation of peripheral blood lymphocytes by phorbol-12-myristate-13-acetate (PMA) leads to increased expression of the T cell receptor CD3 (Figure A, mean from 3 experiments).
For HUVECs (human umbilical vein endothelia cells) the induction of E-selectin (CD62E) expression upon IL-1β treatment is shown (Figure B, white bars) in comparison to results from a conventional flow cytometer (white bars).

Corresponding application note: 5989-2718EN

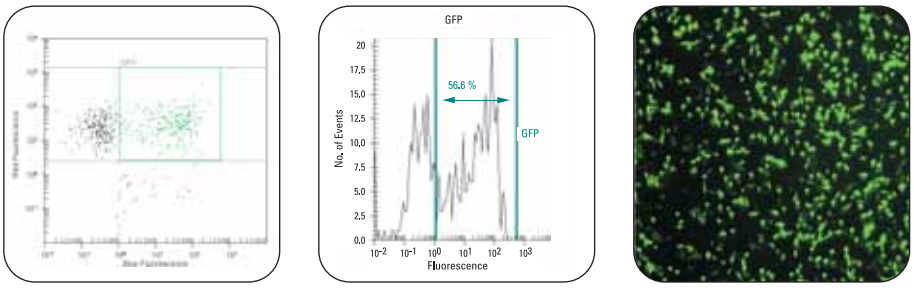
Transfection efficiency monitoring

Green fluorescent protein in CHO cells

Mock transfected cells



GFP transfected cells

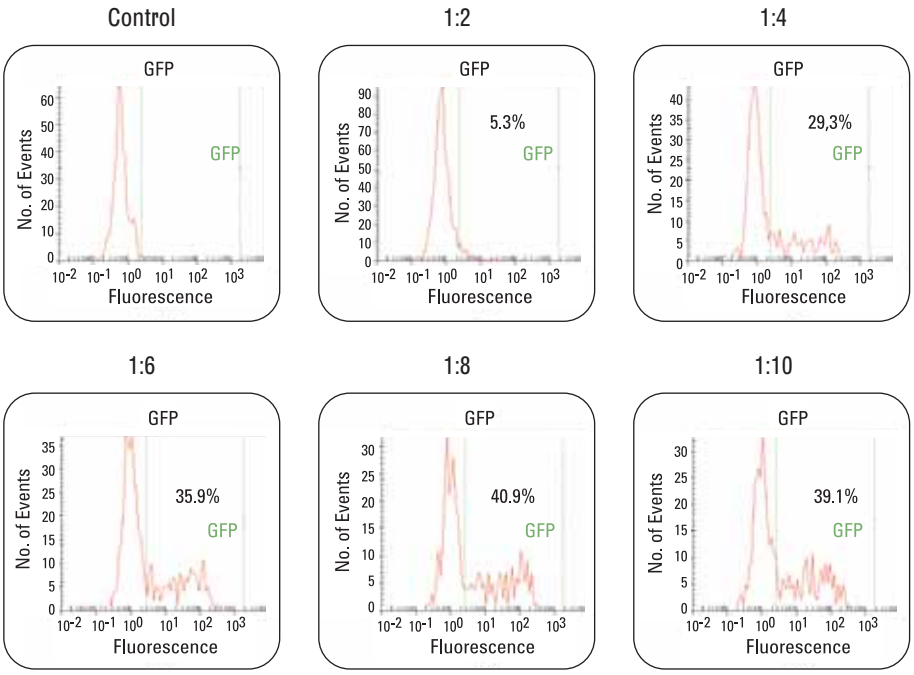


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by a lipofection method. The upper panel shows the control mock transfection; here cells don't express GFP. Examples for data evaluation in dotplot view and histogram view are shown in comparison to the microscopy view. For analysis on the Agilent 2100 bioanalyzer, cells were stained with a red dye for live cells (reference stain). The transfection efficiency of 56% can be easily determined with the Agilent 2100 bioanalyzer.

Corresponding application note: 5988-4320EN

Transfection efficiency monitoring

On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios



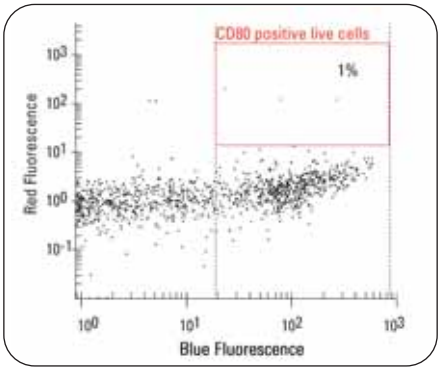
Kit: Cell fluorescence kit
Assay: On-chip GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by alipofection method. Optimization of transfection conditions were done on one chip. Several DNA:lipofectamine ratios were tried. A ratio of 1:8 gave the best transfection efficiency. All cells were reference stained with a red live dye. On-chip staining was applied, minimizing the staining time, reagent usage and cell consumption.

Corresponding application note: 5988-7296EN

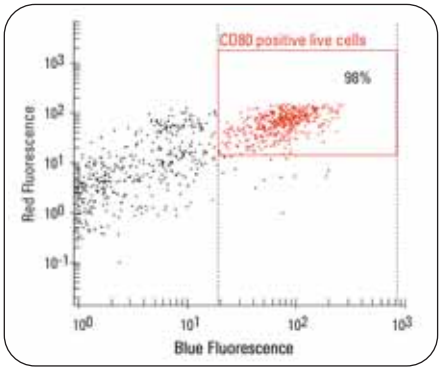
Transfection efficiency monitoring

Verification of stable transfected cell clones by on-chip antibody staining

Hek 293 control cells



CD 80 stable clone

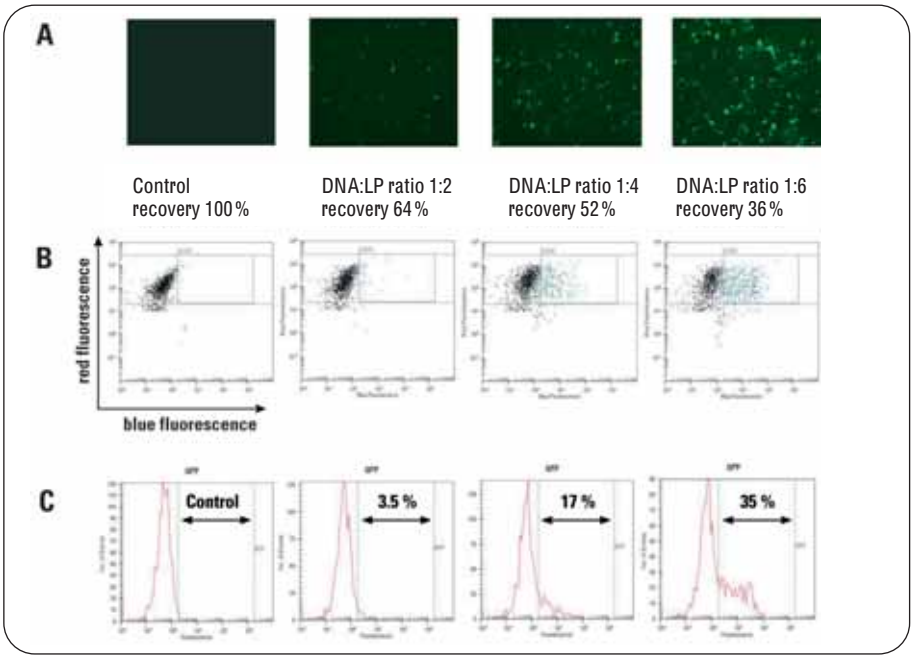


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Verification of CD80 protein expression in stable transfected Hek 293 cells with the Agilent 2100 bioanalyzer. Control (left dot plot) and CD80 transfected cells (right) are stained on-chip with blue calcein live dye and anti-CD80-CyChrome antibody. Red region marks CD80 protein expressing 293 cells within live cell population - confirming expression in the CD80 stable clone Hek 293 cells.

Corresponding application note: 5988-7111EN

Transfection efficiency monitoring

Transfection of primary cells

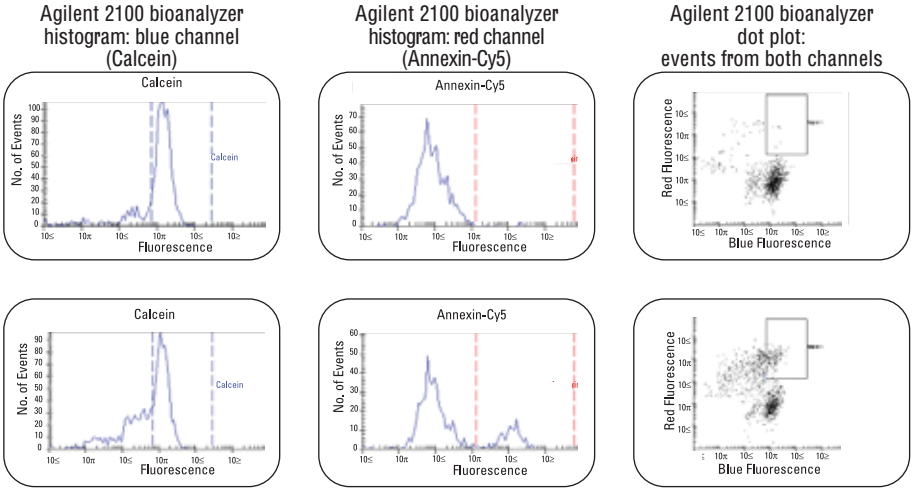


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Monitoring the transfection efficiency in primary cells requires low cell consumption, high reproducibility of results, a fast on-chip staining procedure and ease-of-use all provided by the Agilent 2100 bioanalyzer. The transfection efficiency using a GFP-coding plasmid (pEGFP-C2) at varying plasmid:lipofectamine ratios (DNA:LP ratio) obtained with human umbilical vein endothelial cells (HUVEC) is measured in this optimization series. Images from a fluorescence microscope (A) and dot plots (B), as well as histograms (C) of control- and GFP-transfected cells are shown. Using increasing ratios, better transfection efficiency was achieved, whereas the toxicity of LP caused decreased recovery of living cells. Such data facilitates optimizing transfection conditions.

Corresponding application note: 5988-8154EN

Apoptosis detection

Detection of phosphatidylserine on the cell surface via Annexin V binding

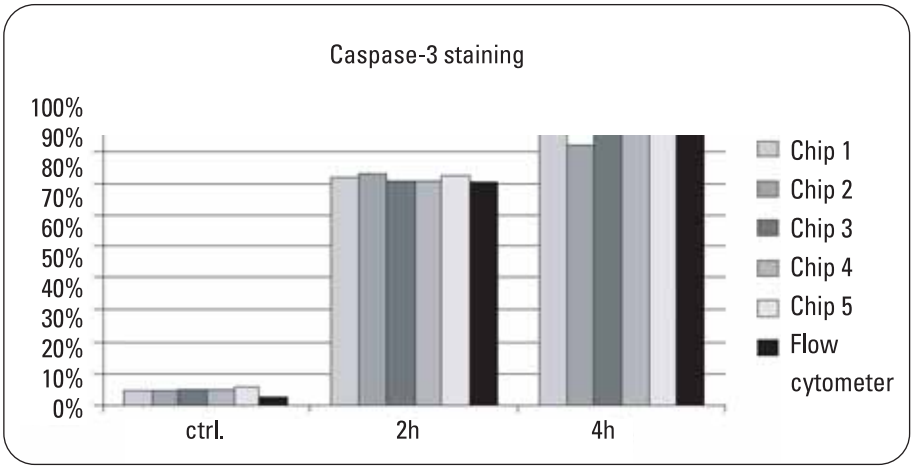


Kit: Cell Fluorescence kit
Assay: Apoptosis assay
Application: Apoptosis (programmed cell death) in Jurkat cells was induced with camptothecin. Cells treated for 16 hours and untreated cells were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin-V binding is made detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as a non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence.

The histograms on the left show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells. The histograms on the right shows all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells. The dot plot of the treated sample nicely shows the subpopulation of all live cells which are apoptotic.
Corresponding application note: 5988-4319EN

Apoptosis detection

Intracellular Caspase-3 antibody staining assay



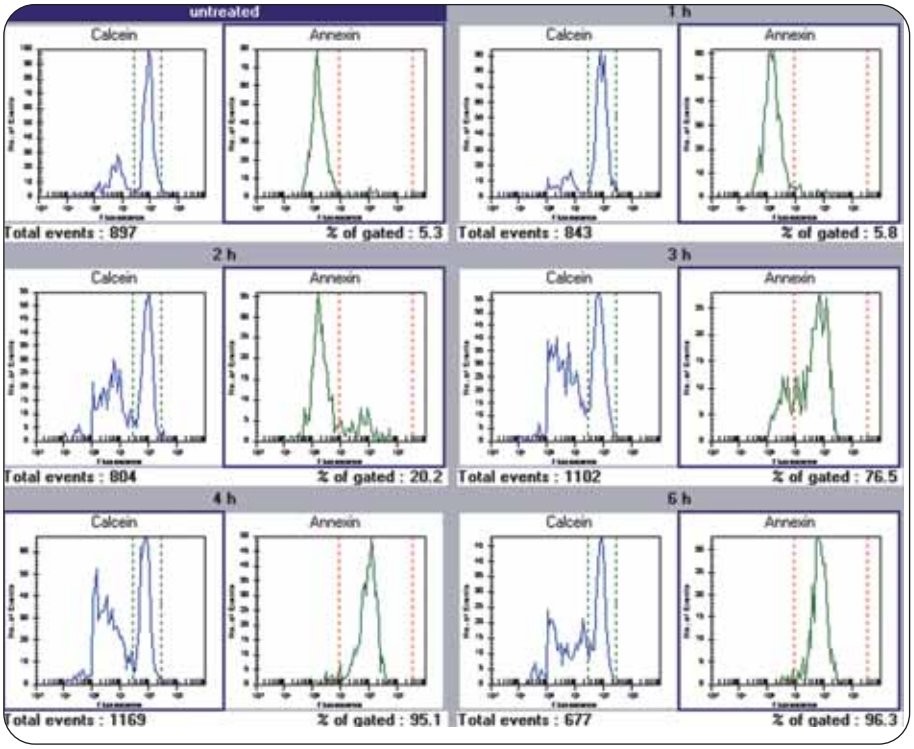
Kit: Cell fluorescence kit
Assay: Generic assay
Application: Induction of apoptosis in Jurkat cells was done with anti-FAS antibody treatment. Intracellular staining with specific antibodies against 'active' Caspase-3 were performed. Reference staining was done with SYTO16 DNA dye. Good chip to chip reproducibility and good comparison to conventional flow cytometer results were obtained.

Corresponding application note: 5988-4319EN

Apoptosis detection

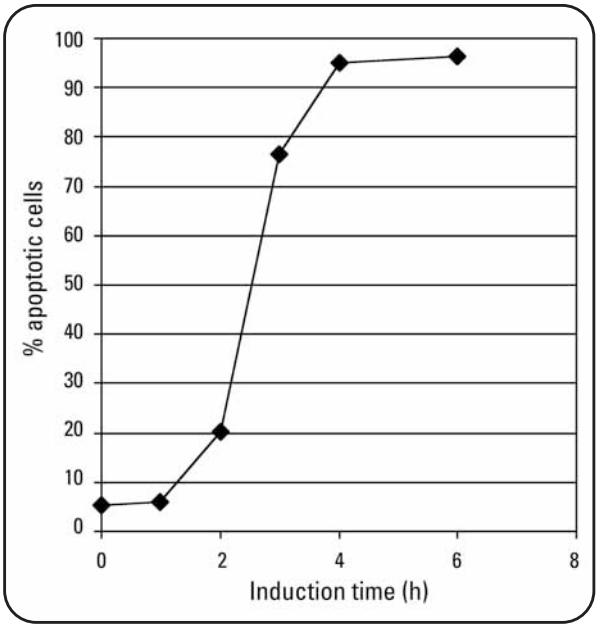
Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody

A



Kit: Cell fluorescence kit
Assay: Apoptosis assay
Application: Apoptosis (programmed cell death) in Jurkat cells was induced with anti-FAS antibody. Cells treated for 0,1,2,3,4 and 6 hours were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin V binding is detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence and indicates apoptosis.

B

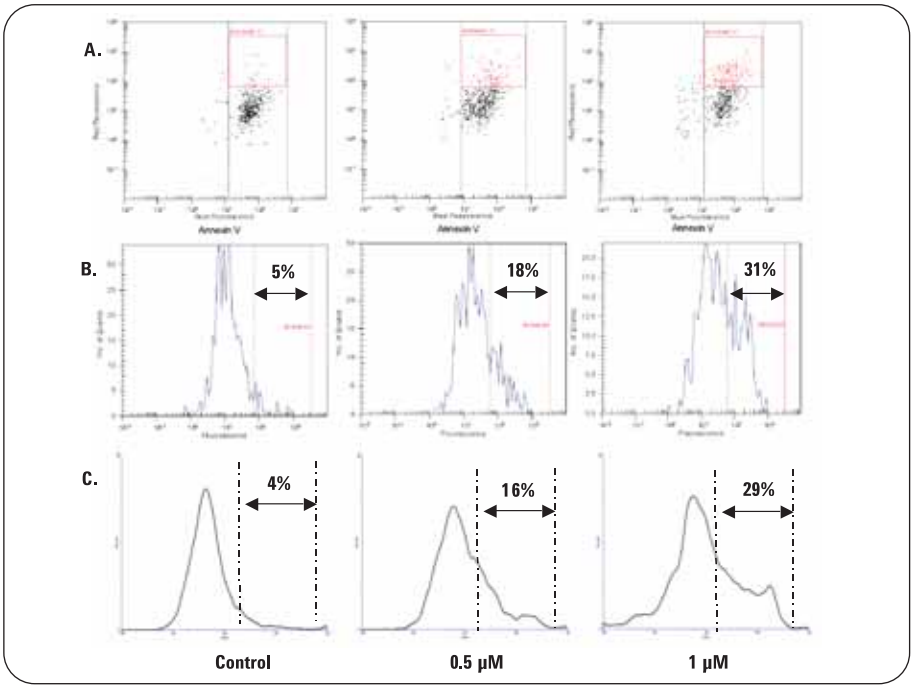


The histograms on page 18 (A) show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells.
The histograms on the right show all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells.
(B) Time course of the induction of apoptosis by anti-FAS antibody in Jurkat cells. Apoptosis is detectable in a significant amount of cells after 2 hours. Following a treatment of 4 hours, approximately 95% of the cells are apoptotic.

Corresponding application note: 5988-4319EN

Apoptosis detection

Apoptosis detection in primary cells



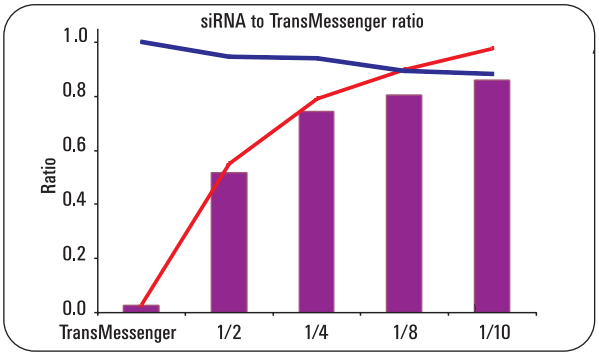
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The Agilent 2100 bioanalyzer has been used to study induced apoptosis by monitoring annexin V-binding in primary human endothelial cells (HUVEC, not shown) and human dermal fibroblasts (NHDF, shown). A simple and fast assay protocol was used on cells left untreated or treated for 5 hours with different concentrations of staurosporine, which induces apoptosis. See row A for dot blots and B for histograms at different concentrations. Evaluation of the same samples on a conventional flow cytometer (row C) yielded similar results.

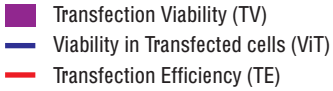
Corresponding application note: 5989-2934EN

Gene silencing in cell culture

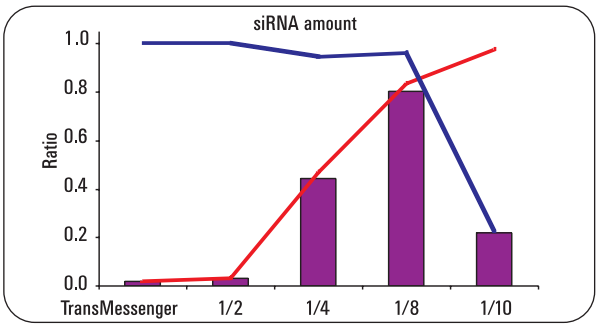
siRNA transfection optimization

A





B

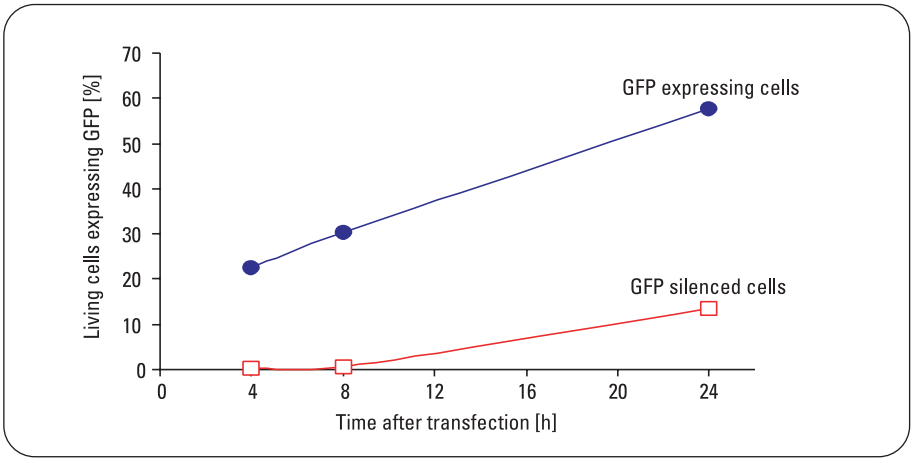


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: In gene silencing experiments (HeLa cells) we found that increasing amounts of transfection reagent (TransMessenger™)to a constant amount of siRNA leads to a plateau of transfection viability (panel A). Transfection viability reflects the product of the viability of the transfected cells and the transfection efficiency. With a constant siRNA/transfection reagent ratio of 1:4 and increasing total amounts of introduced siRNA (panel B) the viability of transfected cells decreases at a certain point although the transfection efficiency increases. Thus, there are experimental conditions where the number of living and transfected cells are at a maximum. The Agilent 2100 bioanalyzer features on-chip staining and leads to excellent results with a minimal consumption of cells and reagents.

Corresponding application note: 5988-9872EN

Gene silencing in cell culture

Monitoring of gene silencing experiments



Kit: Cell fluorescence kit
Assay: GFP Assay
Application: After co-transfection of a GFP plasmid and Cy5-labeled siRNA (GFP-specific), GFP expression and viability of cells were detected. The course of GFP expression in control (GFP only) and siRNA/GFP transfected cells was measured on the Agilent 2100 bioanalyzer. Accurate results were obtained fast and in an automated manner. They easily allow the efficiency and reliability of a given protocol and transfection reagents to be judged. Thus, such an experiment provides efficient monitoring and optimization of any gene silencing experiment.

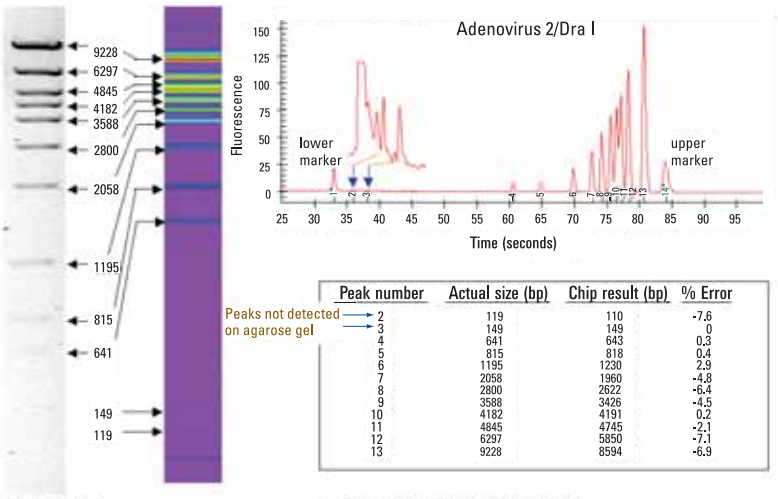
Corresponding application note: 5989-0103EN

II. DNA analysis

	Agriculture/Food	Drug Discovery/Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Restriction digest analysis							
Sizing range exemplified by the separation of Adenovirus 2/Dra I				●			
Detection of single base mutations (I)				●			●
Detection of single base mutations (II)				●			●
PCR product analysis							
Separation of 3 different mixtures of PCR products				●			●
Determination of PCR product impurity				●			●
Multiplex PCR analysis of bacteria in chicken	●			●			●
Multiplex PCR with 19 products	●			●			●
Gene expression analysis							
mRNA expression study by comparative multiplex PCR	●	●		●			
Standardized end-point RT-PCR	●	●		●			
Co-amplification of GAPDH and hsp72				●			
Co-amplification of GAPDH and hsp72 - response curves				●			
Competitive PCR	●			●			●
Food analysis							
Development of meat specific assays (I)	●			●			
Development of meat specific assays (II)	●			●			
Fish species identification by RFLP	●			●			
GMO detection							
Development of a multiplex assay for soya	●			●			
DNA stability during food processing	●			●			
GMO detection by nested multiplex PCR	●			●			
Oncology							
Tumor cell detection from carcinoma patient blood		●		●			
SNP analysis in cancer related P16 gene		●		●			
K-ras gene SNP detection		●		●			
METH-2 downregulation in lung carcinomas		●		●			
Label-free analysis of microsatellite instability in carcinoma		●		●			
Diagnostic research							
Genotyping of H. pylori	●	●		●			●
Duplications and deletions in genomic DNA		●		●			
Forensic testing							
Optimization of PCR on mtDNA				●			●
Pitfalls in mtDNA sequencing				●			●

Restriction digest analysis

Sizing range exemplified by the separation of Adenovirus 2/Dra I



Peak number	Actual size (bp)	Chip result (bp)	% Error
2	119	110	-7.6
3	149	149	0
4	641	643	0.3
5	815	818	0.4
6	1195	1230	2.9
7	2058	1960	-4.8
8	2800	2622	-6.4
9	3588	3426	-4.5
10	4182	4191	0.2
11	4845	4745	-2.1
12	6297	5850	-7.1
13	9228	8594	-6.9

Agarose gel

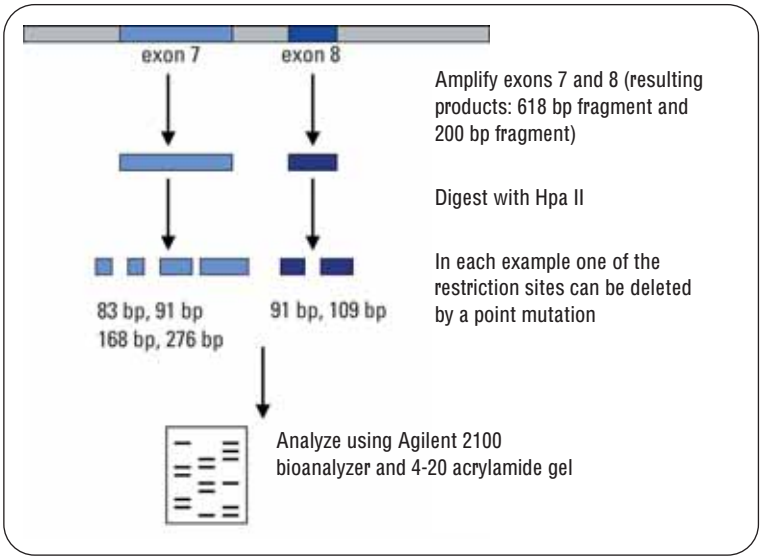
Agilent 2100 bioanalyzer data

Kit: DNA 12000 kit
Assay: DNA 12000 assay
Application: Restriction digest analysis of Adenovirus 2/Dra I. For restriction fragment analysis the large linear dynamic range of the lab-on-a-chip approach is very advantageous. Analyzing samples with large and short fragments on slab gels can be difficult because of bands running off the gel and insufficient staining (or over-staining) of bands.

Corresponding application note: 5968-7501EN

Restriction digest analysis

Detection of single base mutations (I)

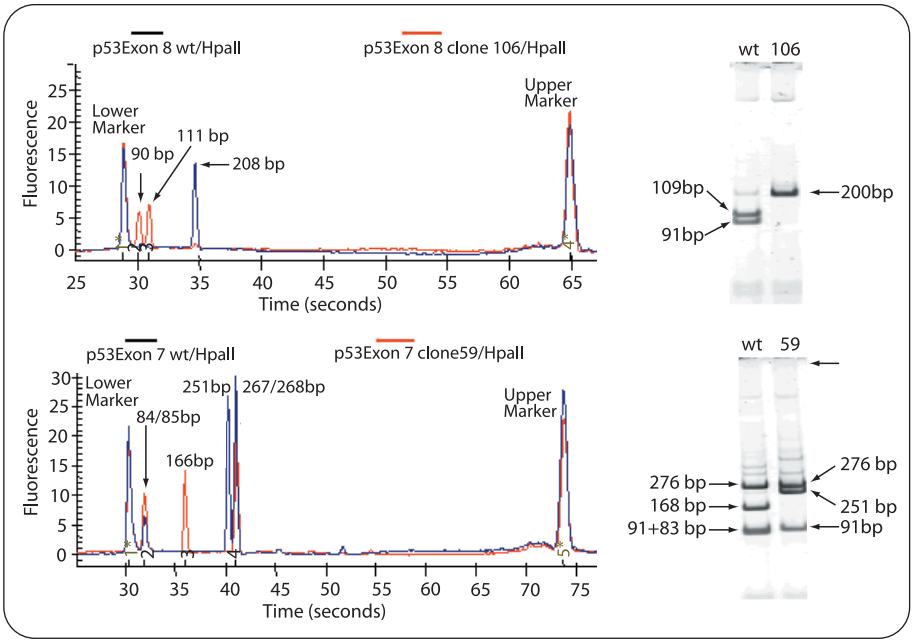


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Mutation detection by RFLP highlights the use of the Agilent 2100 bioanalyzer. Two different regions of the p53 gene were amplified with specific primers and digested with Hpa II, which cuts in a location that is prone to mutations. In the presence of a point mutation, the enzyme Hpa II does not cleave the DNA, leaving larger fragments that can be revealed by gel electrophoresis or by analysis with the DNA 7500 LabChip kit (see next page).

Corresponding application note: data not published

Restriction digest analysis

Detection of single base mutations (II)

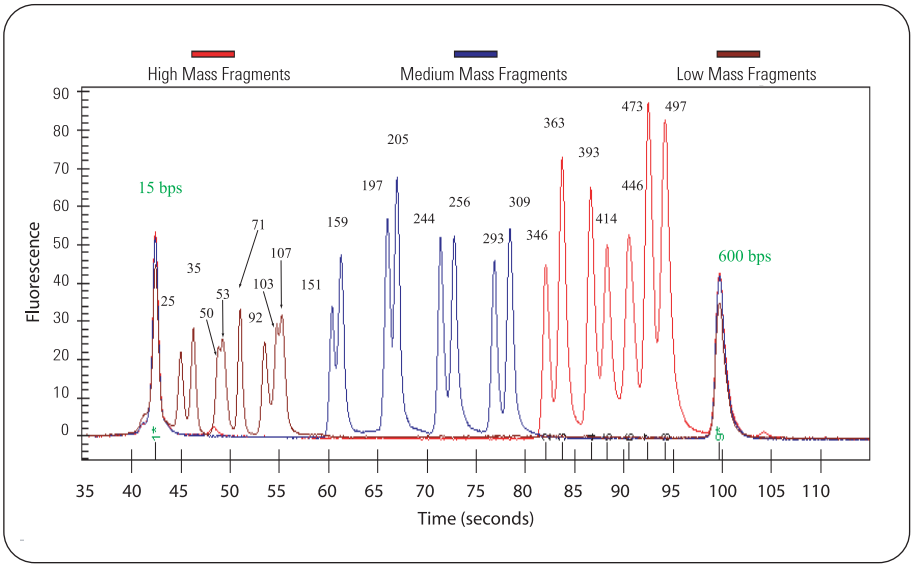


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Analysis on the chip showed an identical pattern of digest fragments as seen on the slab gel for the wildtype and Exon 7 & 8 PCR products. Comparison of the calculated sizes of the bands shows 1-2% variance with the LabChip assay, which allows fast and accurate detection of point mutations.

Corresponding application note: 5968-7496EN

PCR product analysis

Separation of 3 different mixtures of PCR products

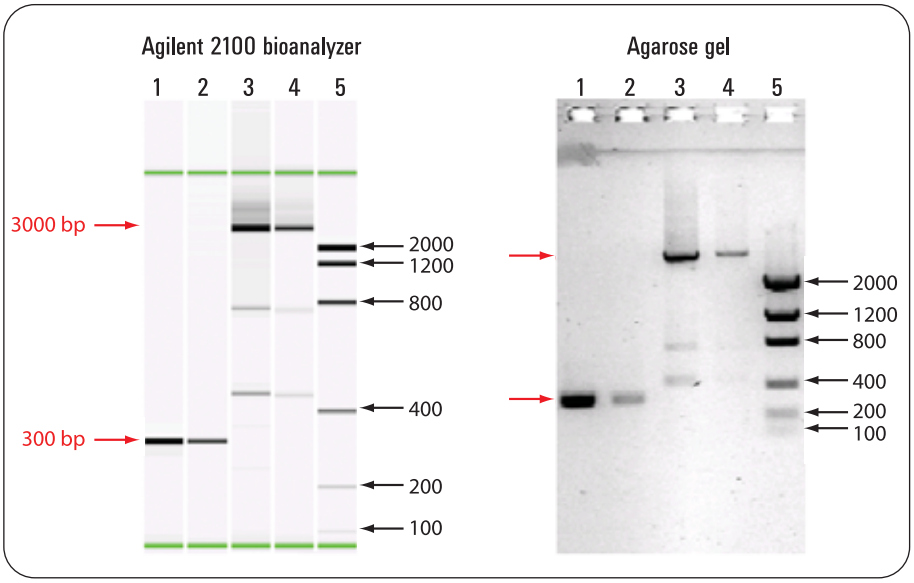


Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Overlay of three different electropherograms, which are mixtures of PCR samples ranging from 25 to 500 base pairs in size. The two closest eluting bands (50 bp and 53 bp) are partially separated and identified by the software as two separate peaks. The DNA 500 assay achieves a resolution of five base pairs from 25 to 100 base pairs and a 5% resolution from 100 to 500 base pairs where the sizing error is less than 10% over the entire size range.

Corresponding application note: 5988-3041EN

PCR product analysis

Determination of PCR product impurity

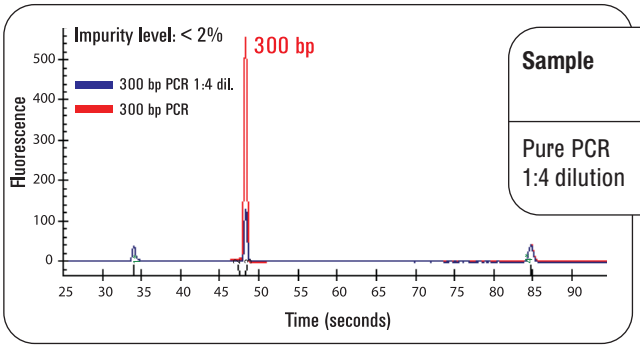


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Comparison between the analysis of two PCR reactions (300 and 3000 bp products) using the DNA 7500 LabChip kit vs. an agarose gel. Two different concentrations are shown side by side for each PCR reaction (undiluted and 1:4 dilution). The Agilent 2100 bioanalyzer shows superior performance in locating impurities over a broader concentration range than the gel. The 300 bp fragment appears to be uncontaminated in both the gel and on the Agilent 2100 bioanalyzer. The 3000 bp fragment shows few impurities on the gel, which become invisible at the 1:4 dilution. These impurities can easily be detected with the Agilent 2100 bioanalyzer.

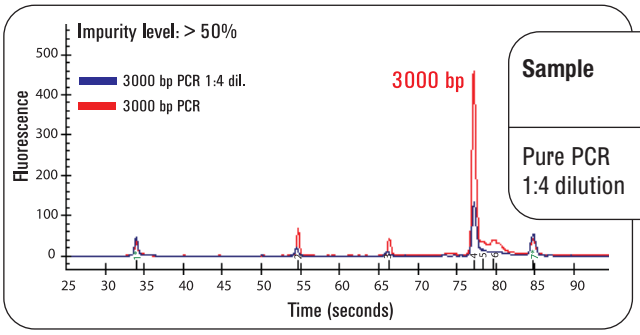
Corresponding application note: 5968-7496EN

PCR product analysis

Determination of PCR product impurity



Sample	c (DNA) All peaks	Main peak (300bp)
Pure PCR	41.4 ng/µl	40.7 ng/µl
1:4 dilution	9.6 ng/µl	9.6 ng/µl



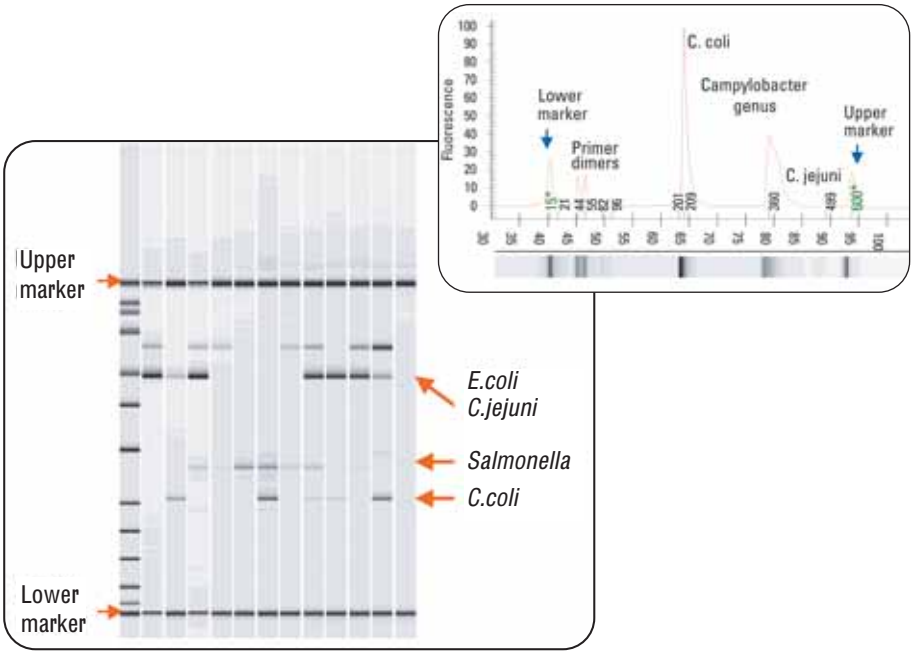
Sample	c (DNA) All peaks	Main peak (3000bp)
Pure PCR	61.9 ng/µl	40.7 ng/µl
1:4 dilution	14.8 ng/µl	9.8 ng/µl

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: The quantitative data generated by the Agilent 2100 bioanalyzer indicate the amount of impurity or non-specific products in the PCR reactions from the previous page. Even in the 300 bp fragment a small impurity can be detected, while the 3000 bp fragment shows more than 50% impurities.

Corresponding application note: 5968-7496EN

PCR product analysis

Multiplex PCR analysis of bacteria in chicken



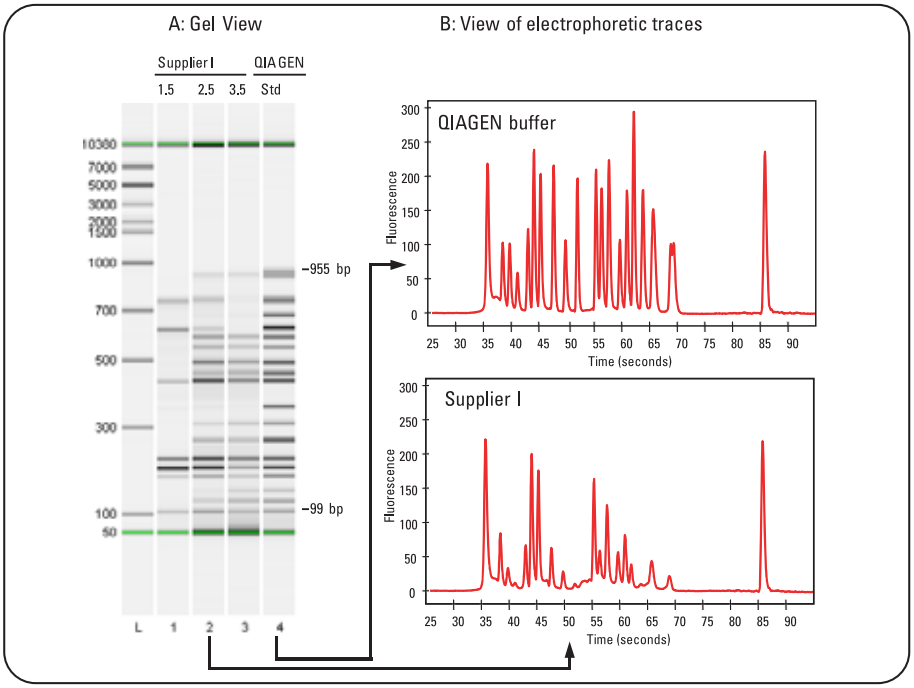
Data kindly provided by GenPoint, NL

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex PCR with four primer pairs, each one specific for a certain DNA sequence from one of the 4 bacteria to be tested for. Total DNA was extracted from chicken and subjected to PCR. The gel-like image shows traces from different chicken samples with bands showing up when an amplicon could be detected. The electropherogram is one example where bacterial DNA from two species of the *Campylobacter* genus could be detected.

Corresponding application note: data not published

PCR product analysis

Multiplex PCR with 19 products



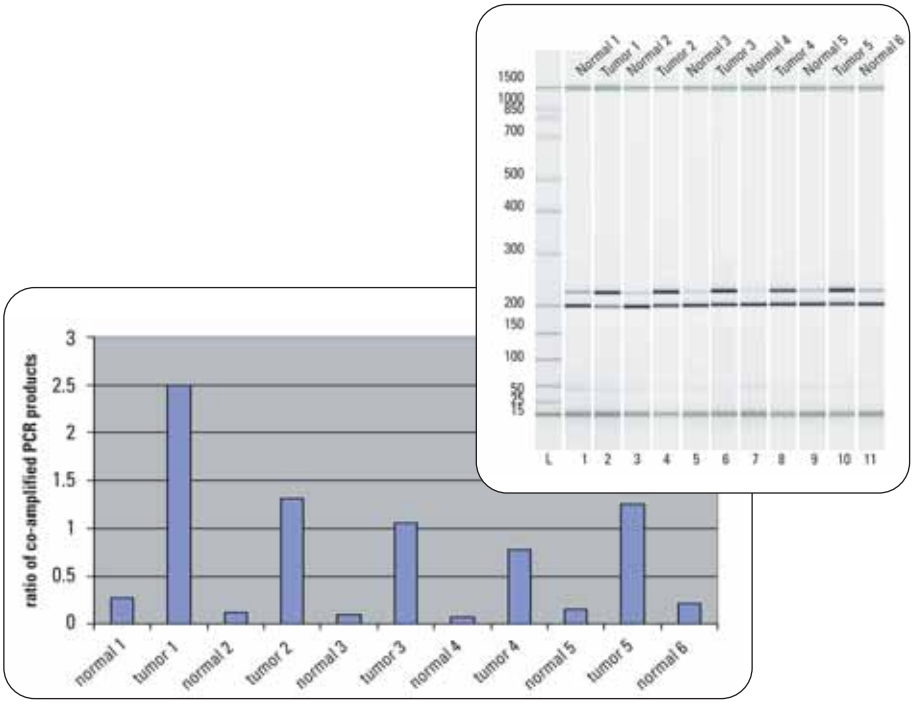
Data kindly provided by Qiagen, Germany

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Many molecular applications include PCR multiplexing as shown above with a PCR that yields 19 products. Applications are genotyping of transgenic organisms, detection of pathogens or GMs and microsatellite genotyping (e.g. short tandem repeat (STR) and variable number tandem repeat (VNTR) analyses). The sample shows optimization of PCR conditions (Mg^{2+} concentration) performed to ensure annealing of the multiple primers under identical conditions. Visualization and evaluation of the results can be performed efficiently with the Agilent 2100 bioanalyzer because of the high resolution, the accurate sizing, quantitation and extended linear range.

Corresponding application note: 5988-9342EN

Gene expression analysis

mRNA expression study by comparative multiplex PCR



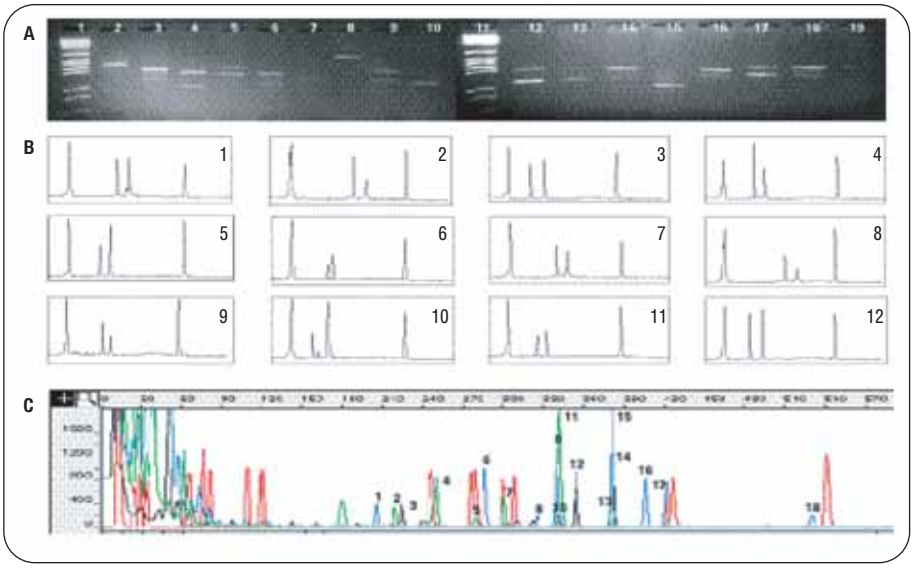
Data kindly provided by the Roy Castle Centre

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were co-amplified in this study. A tumor specific gene (upper band) along with a housekeeping gene (lower band). The upregulation of the tumor gene is visualized via analysis on the Agilent 2100 bioanalyzer. Building the ratio of the concentration values obtained from the Agilent 2100 bioanalyzer, numerical values are obtained that are normalized with regard to the RT-PCR amplification efficiency. This way tumor tissue can be distinguished from normal tissue more unambiguously.

Corresponding application note: data not published

Gene expression analysis

Standardized end-point RT-PCR



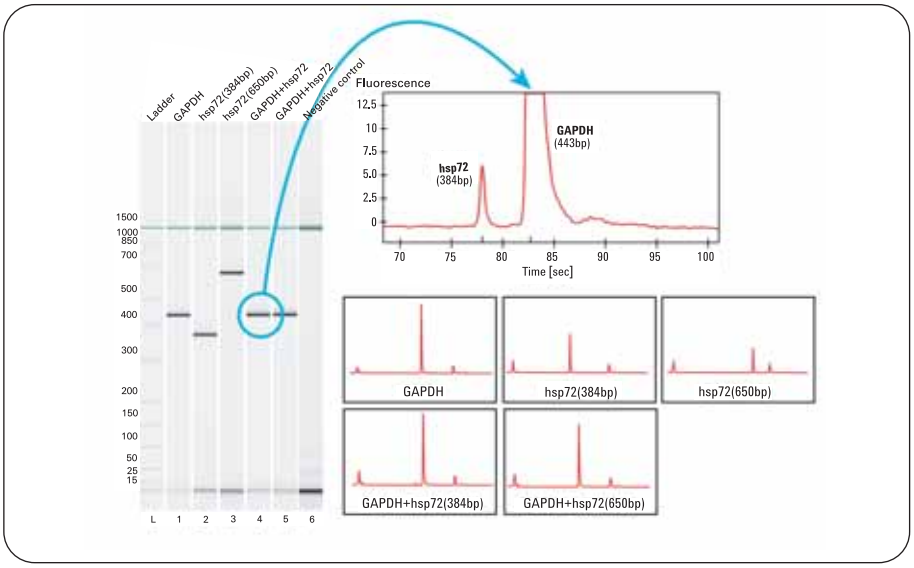
Data kindly provided by the Medical College of Ohio

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Complementary DNA from bronchial epithelial cells (BEC) was analyzed by a Standardized RT-PCR (StaRT) for the expression of 15 different genes. This analysis can be performed at the end-point of PCR without the need for real-time measurement at each cycle of PCR. Three methods for evaluation of representative results were compared (see above). The coefficient of variance (CV) from at least 3 measurements was calculated. The direct comparison of the reproducibility for agarose gel analysis (A, CV = 0.50) and the ABI Prism310 Genetic Analyzer (C, CV = 0.39) with the Agilent 2100 bioanalyzer (B, CV = 0.29) reveals that the Agilent 2100 bioanalyzer is superior. It is a reliable and valuable tool in quantitative gene expression analysis.

Corresponding application note: 5988-3674 EN

Gene expression analysis

Co-amplification of GAPDH and hsp72



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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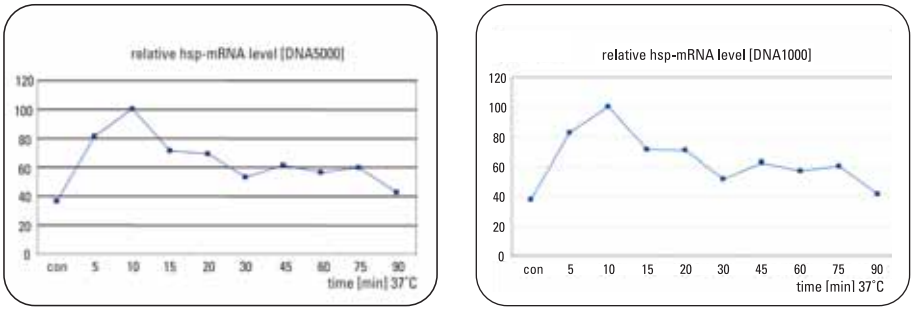
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Gel-like image and electropherograms showing the results of separate amplifications and co-amplifications of GAPDH and hsp72 in unstimulated HepG2 cells. Primers for GAPDH yield a PCR product of 443 bp (lane 1), primers for hsp72 yield PCR products of 384 and 650 bp (lane 2 and 3). Lane 4 and 5 show the results of the co-amplification reactions. Due to the competitiveness of the reaction, very little hsp72 products could be detected in lane 4 (insert) and no product was detected in lane 5 (lane 6 = negative control). The broad linear dynamic range of the analysis allows detection of weak bands next to strong bands and helped in the determination of gene expression in this case.

Corresponding application note: 5988-4556EN

Gene expression analysis

Co-amplification of GAPDH and hsp72 - response curves



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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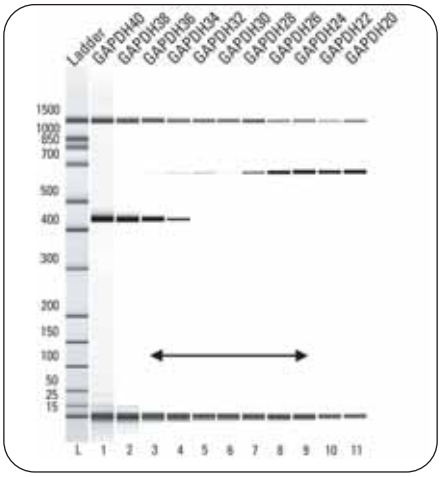
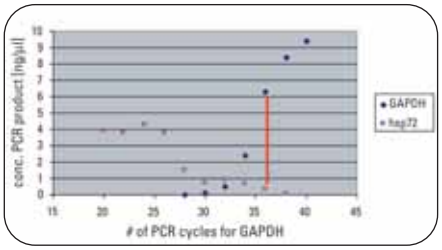
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: The optimized PCR conditions were used to monitor the response of a stimulus to hsp. Gene expression was monitored by comparing the RT-PCR amplification of a housekeeping gene with the co-amplification of hsp. In the current case, the highest gene expression was measured after about 10 minutes. As a comparison, the same set of samples was analyzed using the DNA 500 kit. Virtually identical results are obtained with both kits, demonstrating thatlab-on-a-chip technology can serve as a standardized approach to gel electrophoresis.

Corresponding application note: 5988-4556EN

Gene expression analysis

Competitive PCR





Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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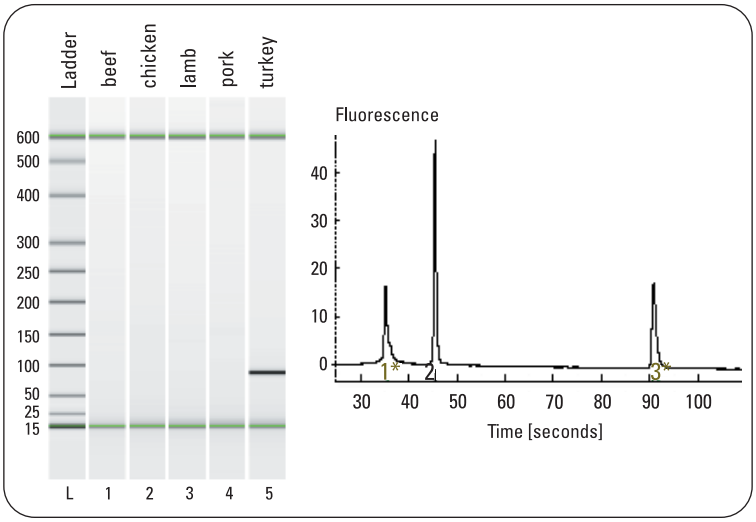
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were reverse transcribed and co-amplified in one reaction tube. The PCR products were analyzed using the DNA 1000 LabChip kit. Primers for hsp72 were present from the beginning of the PCR reactions, while primers for GAPDH were added after various cycle numbers ranging from 20 to 40 cycles (primer dropping method). This allowed optimization of this competitive PCR reaction. The left graph displays the dynamic range (arrow) in the gel like view, whereas the right graph indicates conditions with greatest sensitivity (red line).

Corresponding application note: 5988-4556EN

Food analysis

Development of meat specific assays (I)



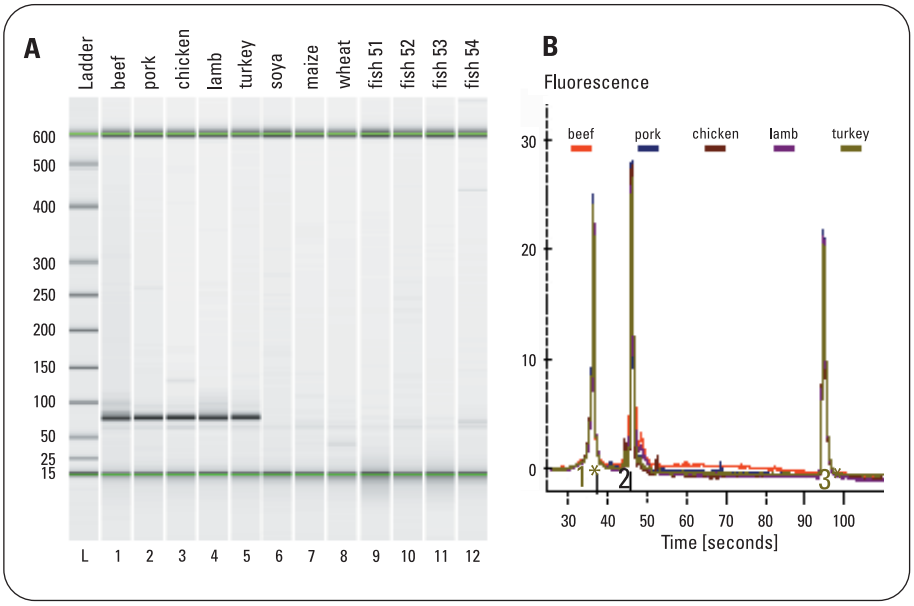
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual species in processed food, PCR assays with specific sets of primers can be developed. Example: turkey specific primers do not amplify any other meat species, including beef, chicken, lamb, or pork (see lane 5 and respective electropherogram).

Corresponding application note: 5988-4069EN

Food analysis

Development of meat specific assays (II)



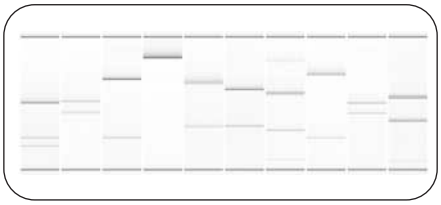
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual component types in processed food, PCR assays with specific sets of primers can be developed. Example: Primers that amplify any type of meat, but do not amplify other food constituents, including soya, maize, wheat or fish.

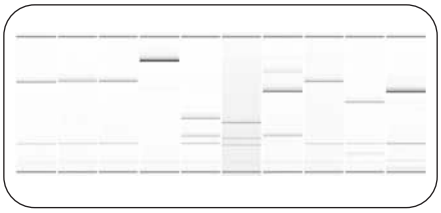
Corresponding application note: 5988-4069EN

Food analysis

Fish species identification by RFLP



Restriction enzyme: DdeI



Restriction enzyme: HaeIII

Restriction enzyme: NlaIII

Common name (UK)	Latin name
Atlantic Cod	Gadus morhua
Pacific Cod	Gadus macrocephalus
Coley (Saithe)	Pollachius virens
Haddock	Melanogrammus aeglefinus
European Hake	Merluccius merluccius
South African Hake	Merluccius paradoxus
European Plaice	Pleuronectes platessa
Whiting	Merlangus merlangus
Alaskan (Walleye) Pollock	Theragra chalcogramma
Hoki	Macruronus novaezelandiae
Atlantic Salmon	Salmo salar
Red / Sockeye Salmon	Oncorhynchus nerka
Pink / Humpback Salmon	Oncorhynchus gorbuscha
Chinook Salmon	Oncorhynchus tschawytscha
Coho / Silver Salmon	Oncorhynchus kisutch
Keta / Chum Salmon	Oncorhynchus keta
Cut-throat Trout	Oncorhynchus clarki clarki
Dolly Varden	Salvelinus malma malma
Cherry Salmon	Oncorhynchus masou masou

Data kindly provided by CCFRA, UK

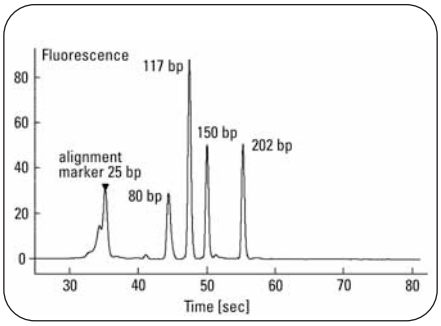
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Identification of white fish and salmon species in the processed state presents a challenge. However, evaluation of PCR-RFLP profiles (PCR-restriction fragment length polymorphism) of a 464 bp region from the cytochrom b gene cut separately with three restriction enzymes facilitated the differentiation of 19 commercially important species. Analysis of the restriction digests was performed with the Agilent 2100 bioanalyzer. This approach was successfully tested in an interlaboratory study.

Corresponding application note: 5989-2982EN

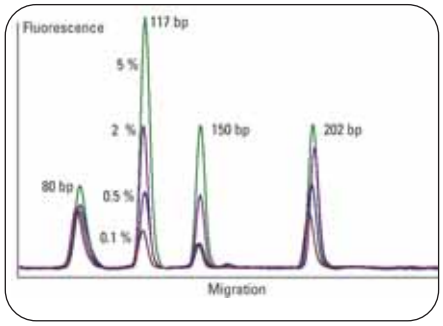
GMO detection

Development of a multiplex assay for soya

A



B



Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex assay for genetically modified (GM) soya. The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled three GM soya targets and one control to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate- 3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya. Figure A: Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit. Figure B: Analysis of certified reference materials containing known amounts of GM soya.

Corresponding application note: 5988-4070EN

GMO detection

DNA stability during food processing

Time at 100°C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

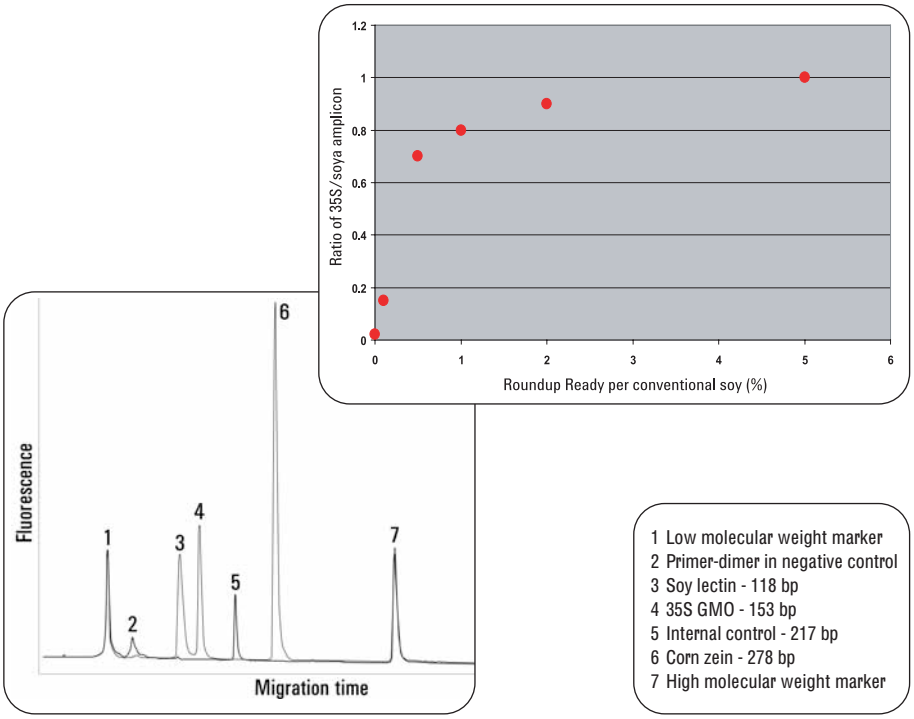
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: The multiplex PCR assay was applied to soya flour samples containing approx. 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (Table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts of products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Corresponding application note: 5988-4070EN

GMO detection

GMO detection by nested multiplex PCR

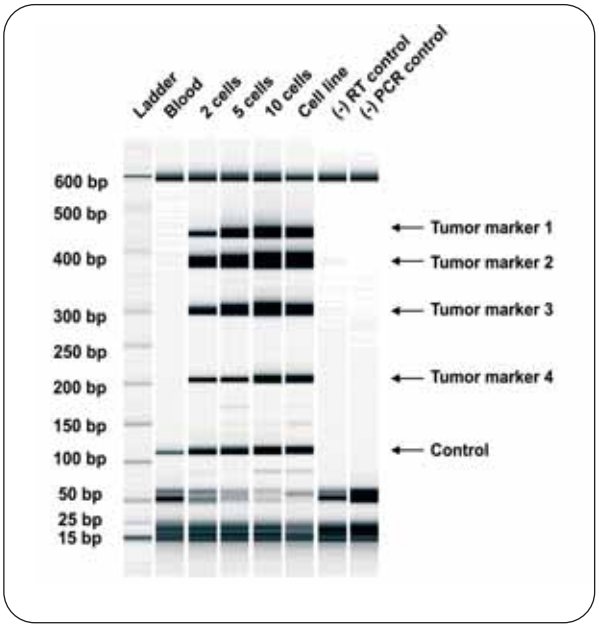


Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: GMO detection by multiplex PCR is widely used for soy and corn. Often sequences from the transgene and species specific controls or internal standard are co-amplified by endpoint PCR in a screening procedure. Multiple products can be analyzed with the Agilent 2100 bioanalyzer at high resolution and sensitivity. Quantification and comparison of product amounts may already lead to qualification of a positive screening result prior to analysis by expensive quantitative real time PCR.

Corresponding application note: 5989-0124EN

Oncology

Tumor cell detection from carcinoma patient blood



Data kindly provided by AdnaGen

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: A combined method of specific tumor cell enrichment and a high sensitivity tumor cell detection by multiplex PCR allows analysis of several tumor marker genes. The method is so sensitive that it allows the detection of only a few tumor cells per 5 ml EDTA-blood. The Agilent 2100 bioanalyzer provides the performance to detect the PCR products with high sensitivity and automated result flagging. This method offers new possibilities for monitoring and prognosis in routine diagnosis, and may facilitate an appropriate selection of patients for adjuvant therapy.

Corresponding application note: 5988-9341EN

Oncology

SNP analysis in cancer related P16 gene

Sample	1	2	3	4	5	6	7	8	9	10	11
Genotype316	C	C	CG	CG	CG	CG	CG	C	C	C	G
Genotype356	C	C	CT	CT	CT	C	C	CT	CT	T	C
gel-like view Agilent 2100 bioanalyzer											
Main Band	198	198	197	198	198	198	198	198	199	198	195
Extra Band 1			204	204	204						
Extra Band 2			216	215	215	214	214				

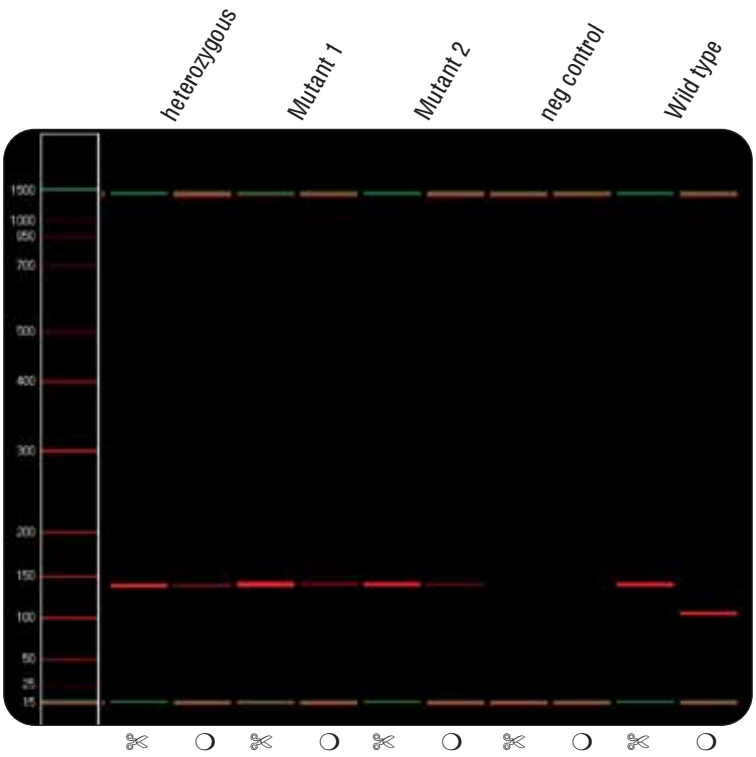
Data kindly provided by SAIC-Frederick

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the exon 3 region of P16 gene are closely related to human cancer. A PCR yields 198 bp fragments with single, expected bands or additional, multiple bands in the Agilent 2100 bioanalyzer analysis. These observations correspond perfectly to genotyping sequencing data of normal and mutant tissues. The pattern of bands is visible due to slower mobility of the heteroduplex formed by heterozygote mutant of the samples. The method provides fast and reliable acquisition of genetic diagnostic data from cancer patients, also on single nucleotide polymorphisms (SNP).

Corresponding application note: 5989-0487EN

Oncology

K-ras gene SNP detection



Data kindly provided by SAIC-Frederick

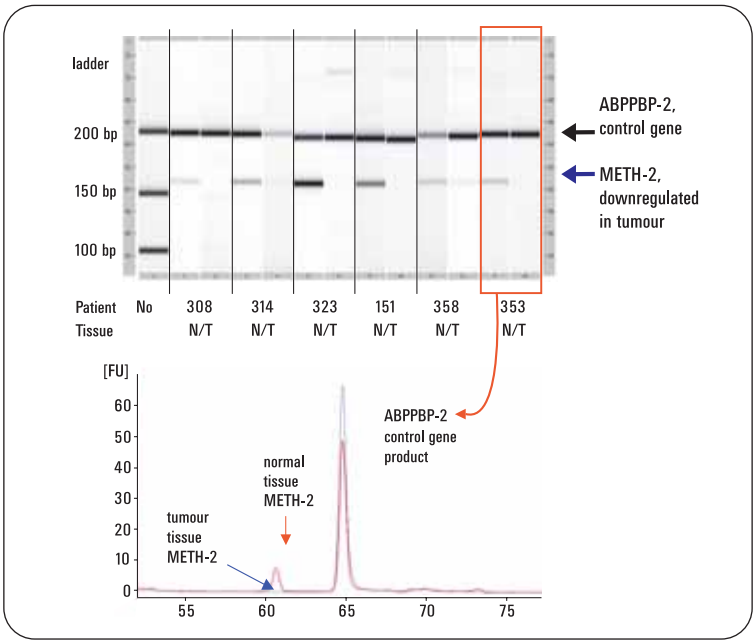
✂ - PCR product
❍ - Restriction

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the K-ras gene coding 12 region can lead to cancer in different human tissues. A dedicated combination of PCR and specific restrictions (BstNI digest) reveals the underlying single nucletide polymorphisms (SNPs). The integral element within this test is the rapid and precise analysis of short amplicons (135 bp, see PCR-product lanes above) and fragments (106 bp, visible in lanes labeled with restriction) with the lab-on-a-chip technique. The test was used to ultimately determine a cancer patient's eligibility for a clinical trial for a peptide vaccine.

Corresponding application note: 5989-0487EN

Oncology

METH-2 downregulation in lung carcinomas



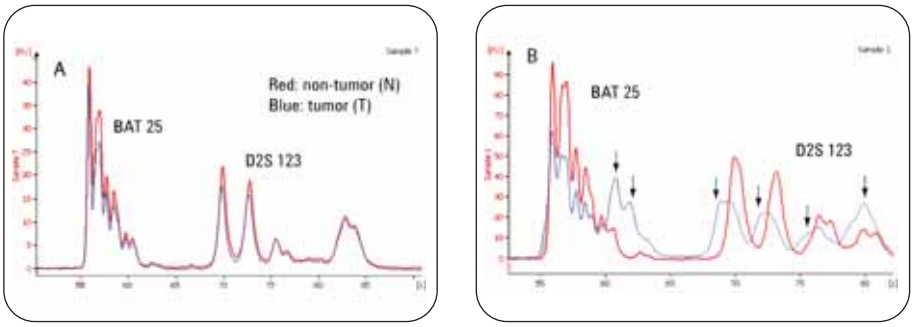
Data kindly provided by Roy Castle Lung Cancer Research Programme,
University of Liverpool, UK

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microarray analysis reveals under- or over-representations of transcripts. Screening of several cell lines for independent validation of such observations can be done with different techniques such as comparative multiplex PCR. This application shows the downregulation of a characteristic antiangiogenetic factor (METH-2) for a series of patient samples. Expression in normal tissue and tissue from the non small lung carcinomas is compared. Results from the array experiments were confirmed on a broad basis. Fast and convenient analysis with the Agilent 2100 bioanalyzer with given quantitation capability fit perfectly in such analytical workflow.

Corresponding application note: 5989-3514EN

Oncology

Label-free analysis of microsatellite instability in carcinoma

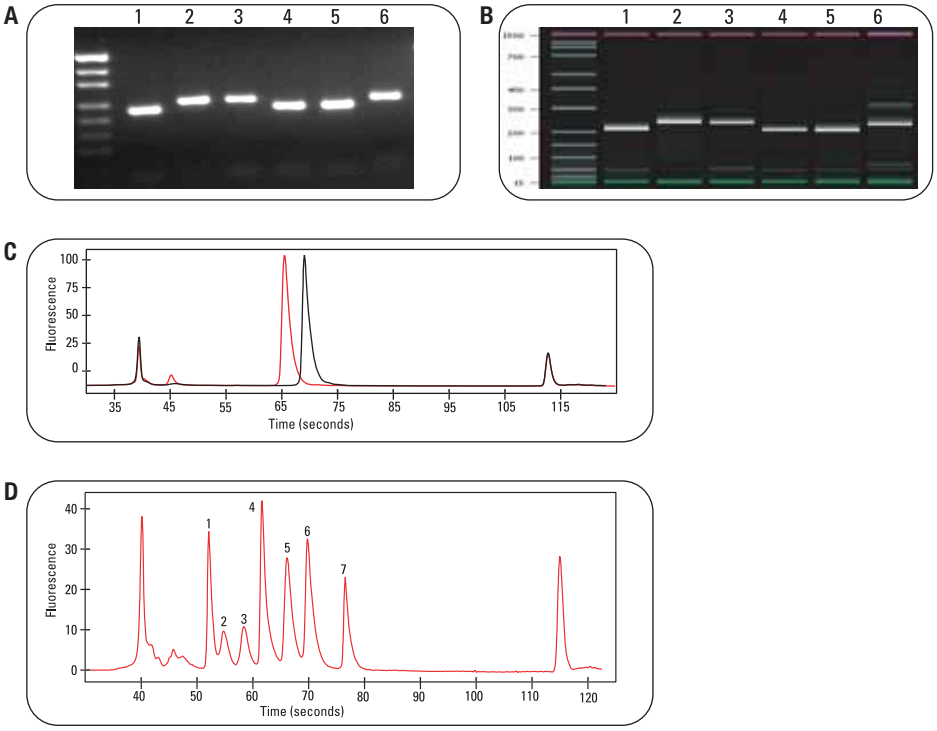


Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system and occurs frequently in various types of cancer. Given that conventional techniques used for MSI detection, for example, polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis, turned out to be laborious or expensive, this study aimed to develop a simple and efficient procedure of MSI detection. Detection of MSI could be demonstrated by microsatellite loci-associated, well defined deviations in the electropherogram profiles of tumor and non-tumor material and confirmed the classification of the MSI cases performed by conventional technology (95% concordance rate). Whereas the results of the MSI detection were comparable to conventional techniques, the on-chip electrophoresis on the Agilent 2100 bioanalyzer was superior in terms of speed, usability and data management.

Corresponding application note: 5989-2626EN

Clinical research

Genotyping of H. pylori



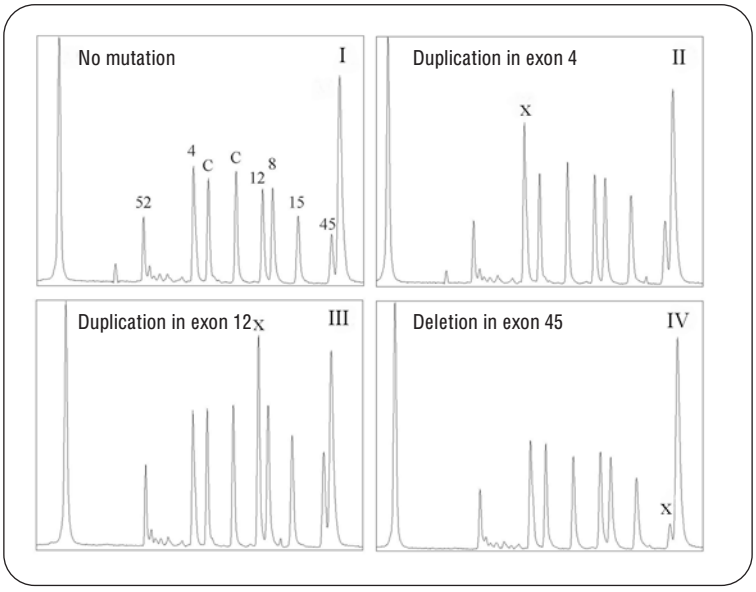
Data kindly provided by Institute for Pathology, Cologne

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Different allelic variants are associated with different stages of H. pylori virulence. Multiplex PCR on five alleles with products in the range of 102 to 301 bp were used to analyze DNA from paraffin embedded tissues. Agarose gel (A) yields only limited distinctiveness, whereas gel-like images (B) and electropherograms (C) show good resolution and superior reproducibility allowing convenient analysis of all desired products in parallel (D). An extended spectrum of prognostic or therapeutic relevant information is now routinely accessible for simultaneous analysis.

Corresponding application note: 5989-0078EN

Clinical research

Duplications and deletions in genomic DNA



Data kindly provided by Center for Human and Clinical Genetics, Leiden

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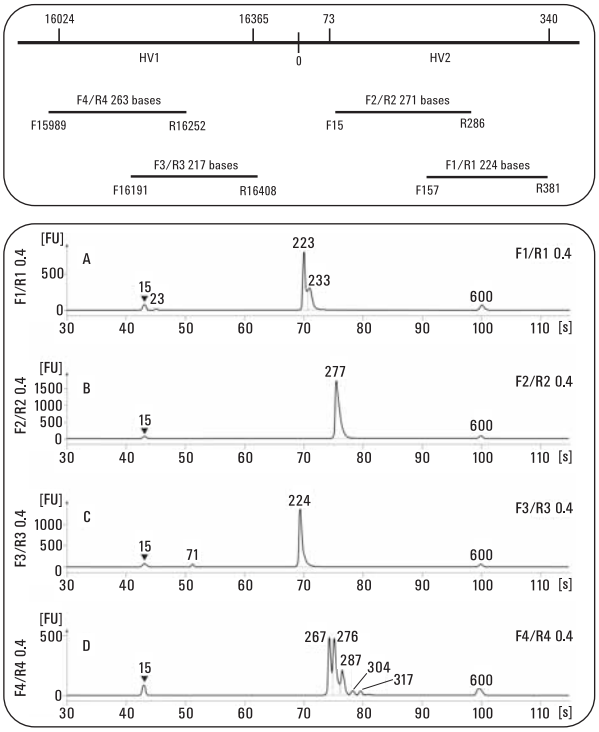
Kit: DNA 500 kit
Assay: DNA 500 assay (in expert software)
Application: Multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent amplification (MLPA) are high throughput techniques for the detection of reordered genomic segments. These methods include hybridization of amplifiable probes with either stringent washing or ligation events prior to amplification. Exact and reproducible sizing and quantitation of multiple products are important prerequisites which are delivered by the Agilent 2100 bioanalyzer and lead to quick and simple analysis of genetically related diseases.

Corresponding application note: 5989-0192EN

Forensic testing

Optimization of PCR on mtDNA



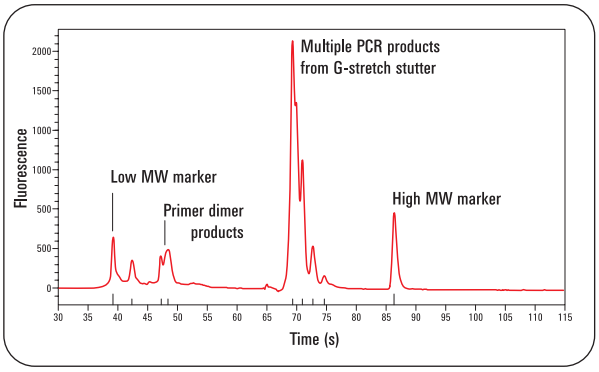
Amplified areas
in human mtDNA

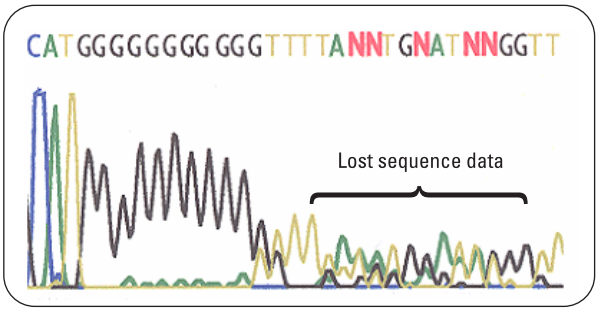
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Human mitochondrial DNA (mtDNA) is amplifiable even from small or badly degraded samples, even if genomic DNA is not available. Lanes B and C show homogenous PCR products which can subsequently be sequenced for identification. However, careful optimization of PCR parameters, like pH, Mg2+ concentration or polymerase amount is necessary and shown in detail in this application note. For example, a high Taq concentration increased the yield but also increased the level of byproducts. PCR for samples in lane D (impurities) and lane A (C-heteroplasmy) need to be improved. The Agilent 2100 bioanalyzer provides a rapid quantitative analysis over the broad size and concentration range needed for optimization and QC. It has proven to be an indispensable tool for forensic labs.

Corresponding application note: 5989-3107EN

Forensic testing

Pitfalls in mtDNA sequencing

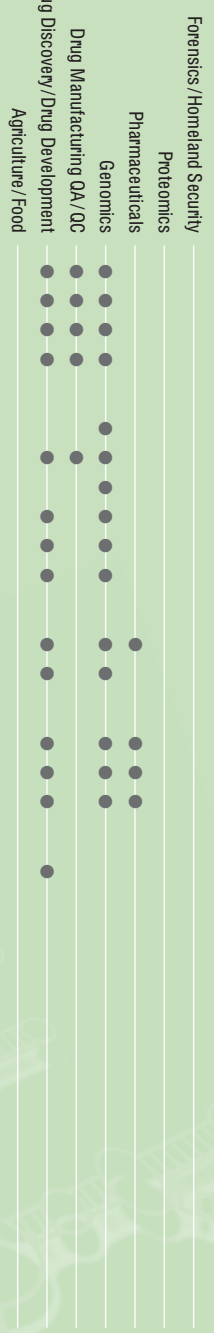




Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Analysis of the non-coding sequence of human mitochondrial DNA (mtDNA) is performed for the purpose of identification in forensics. PCR amplification of limited or degraded mtDNA is done prior to sequencing. Quantitation and quality control of these PCR products (10-100 ng/ml, homogenous fragment in the range of 200-500 bp) was performed. Difficult PCR templates may cause G-stutters or other unintended byproducts of higher or lower mass (left). This may lead to indistinct sequence readings (right). Therefore, e.g. FBI guidelines enforce a 10% impurity level at the most. Fulfillment of this prerequisite can be satisfactorily verified with the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0985EN

III. RNA analysis



Analysis of total RNA
- RNA integrity
- Standardization of RNA Quality Control
- Reproducibility of quantitation
- Genomic DNA contamination

Low amounts of total RNA
- Detection of low levels of RNA
- RNA integrity with the RNA 6000 Pico kit
- RNA quality after staining and microdissection
- Analysis of minimum RNA amounts
- Genomic DNA in low concentrated RNA extracts
- Low RNA amounts from kidney sections

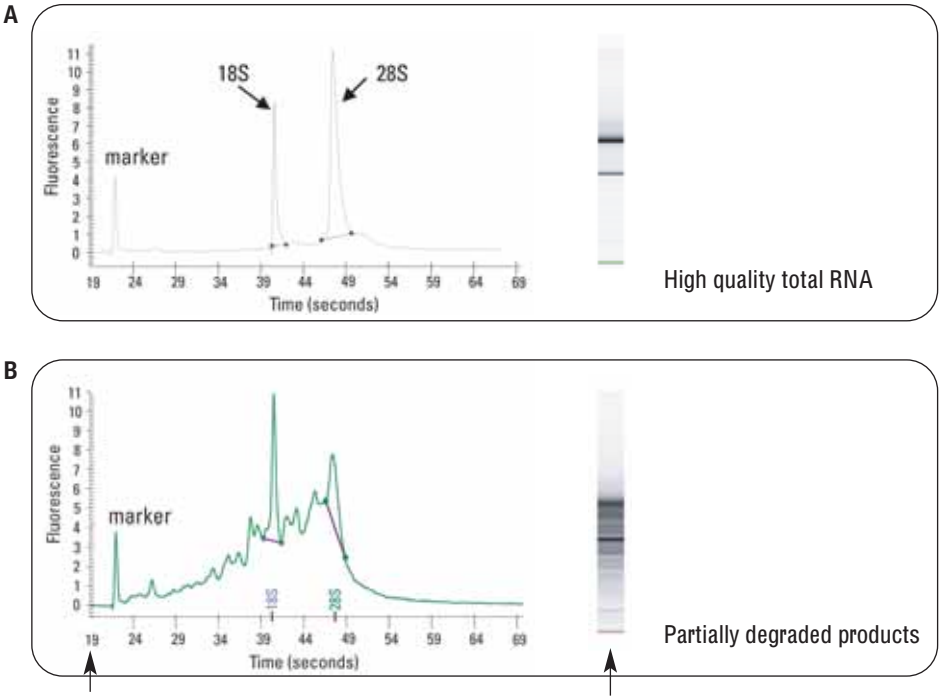
Analysis of mRNA
- RNA integrity
- Ribosomal RNA contamination in mRNA samples

Analysis of Cy5-labeled samples
- Analysis of cRNA with and without dye in gel matrix
- Optimization of labeling reactions
- cRNA fragmentation

Analysis of T7-RNA transcripts
- Size estimation

Analysis of total RNA

RNA integrity

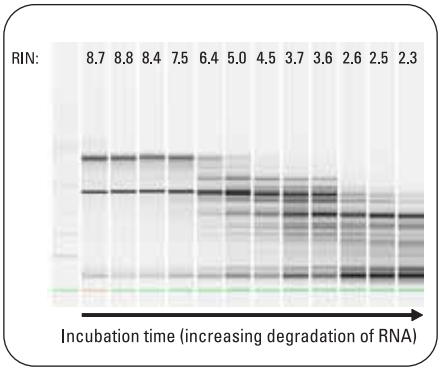


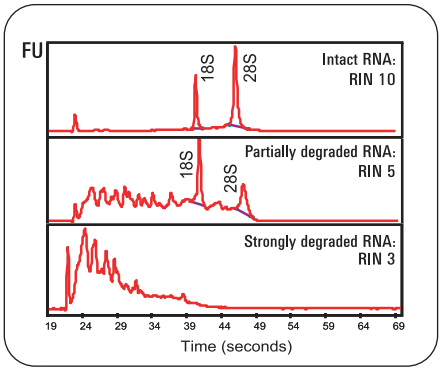
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Analysis of total RNA integrity - a typical first QC step during cDNA or cRNA sample prep for microarrays. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure B shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band. With the help of the Agilent 2100 bioanalyzer and the RNA 6000 Nano kit the important sample QC step prior to an expensive microarray experiment can be easily and quickly achieved.

Corresponding application note: 5968-7493EN

Analysis of total RNA

Standardization of RNA Quality Control





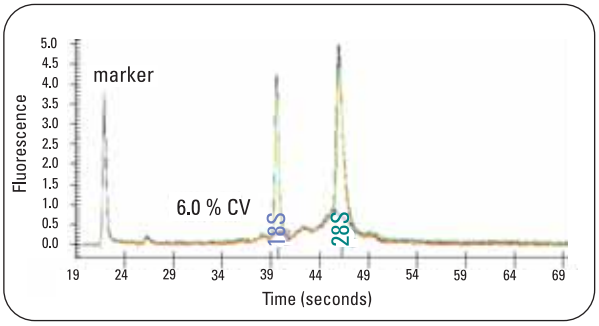
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA integrity number (RIN) is calculated by a dedicated software algorithm (expert 2100 software, starting with Rev 02.01) to assess the quality of RNA preparations. The RIN tool is a major step in the standardization of user-independent RNA evaluation and delivers more meaningful information than simple ratio calculations for ribosomal RNA peaks. It is not influenced by instrument, sample integration and most important, concentration variability, thereby facilitating the comparison of samples and avoiding cost-intensive experiments with low quality RNA preparations. The RIN algorithm is based on a large collection of RNA data of various tissues and qualities. Furthermore, anomalies like genomic DNA contaminations are indicated with weighted error messages (critical/non-critical) to achieve a maximum of reliability.

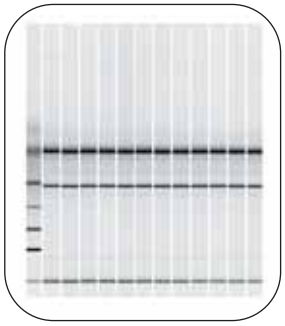
Corresponding application note: 5989-1165EN

Analysis of total RNA

Reproducibility of quantitation

Reproducibility for 12 consecutive runs



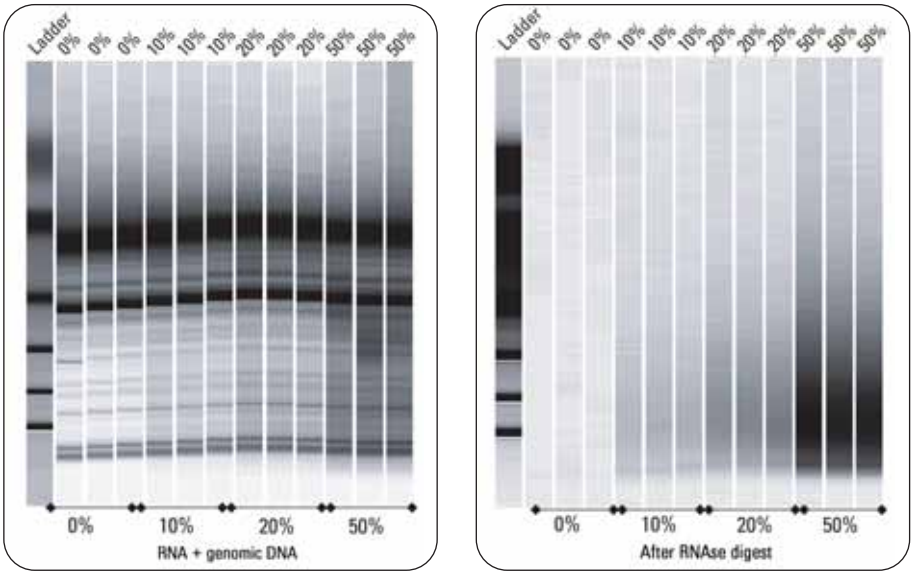


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Alongside the quality control of RNA samples, measurement of RNA concentration is important for (bio-)chemical reactions, such as labeling reactions in the context of microarray experiments. With the RNA 6000 Nano kit good reproducibility can be achieved (here 6% CV), which is little affected by sample contaminants, such as phenol.

Corresponding application note: 5988-7650 EN

Analysis of total RNA

Genomic DNA contamination

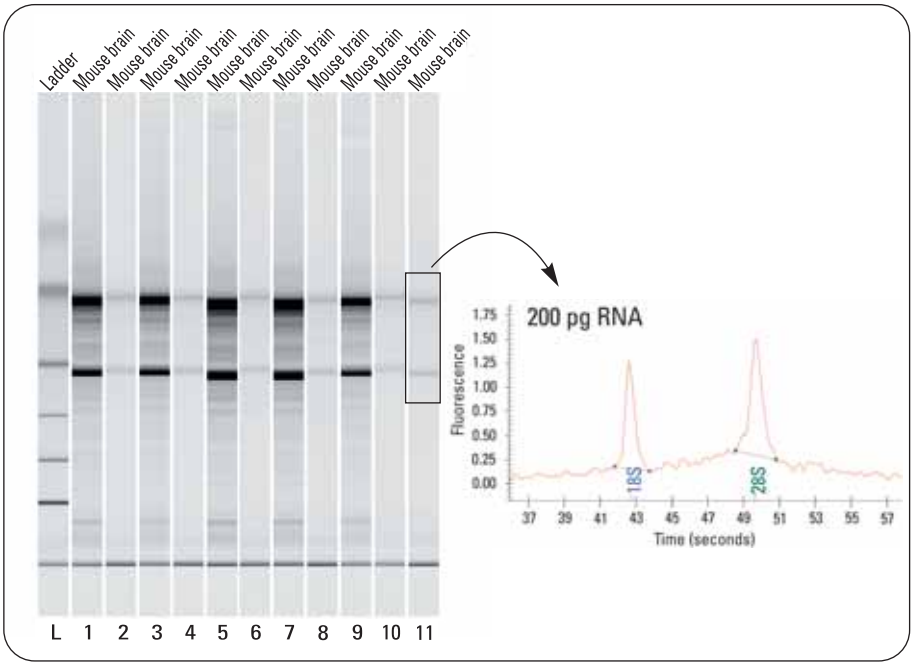


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Gel representation of a chip run with total RNA samples (mouse brain) spiked with varying amounts of herring sperm genomic DNA before and after treatment with RNase. The left panel shows the intact RNA with broad bands in the low MW region stemming from the genomic DNA. After the RNase digest (right panel) only the DNA bands remain, ranging in intensity according to the amount of DNA spiked into the sample.

Corresponding application note: data not published

Low amounts of total RNA

Detection of low levels of RNA



Analysis of mouse brain RNA at two different concentrations

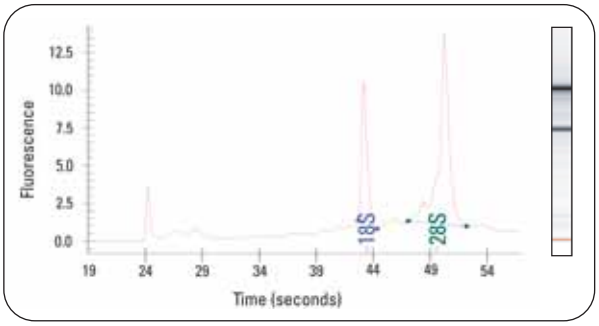
Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: The RNA 6000 Pico kit is complementary to the RNA 6000 Nano kit and is suitable for all applications where the amount of RNA (or cDNA) is limited, e.g. for biopsy samples, samples from microdissection experiments, QC of cDNA made from total RNA, microarray samples, etc. Here Agilent 2100 bioanalyzer results obtained from mouse brain RNA (Ambion) at 200 and 1000 pg/ìl are shown. By analysis in repetitions the reproducibility of quality control is demonstrated. Detection of 200 pg total RNA could be achieved without problems.

Corresponding application note: data not published

Low amounts of total RNA

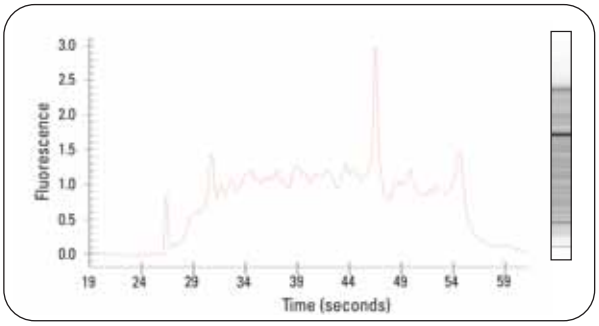
RNA integrity with the RNA 6000 Pico kit

A



Intact RNA

B



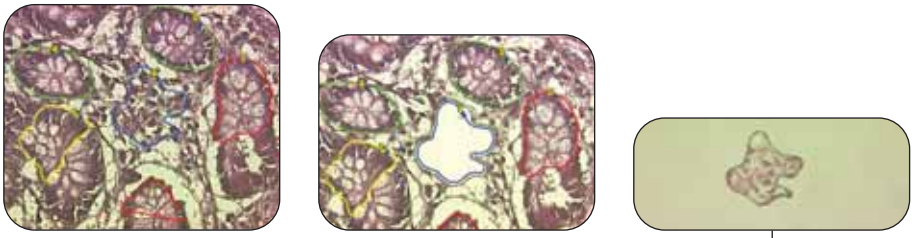
Degraded RNA

Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: Detection of RNA degradation with the RNA 6000 Pico kit. Sample: mouse liver total RNA (Ambion) concentration: 1 ng. Degradation was accomplished by adding a low amount of RNase. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure A shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band.

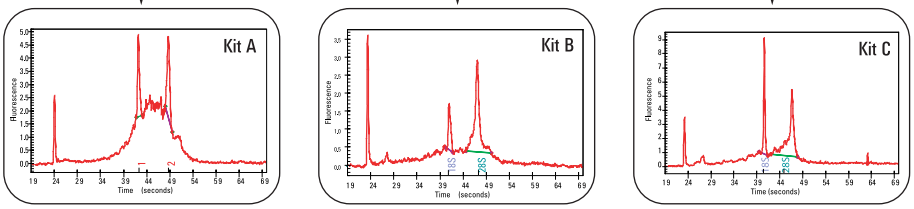
Corresponding application note: data not published

Low amounts of total RNA

RNA quality after staining and microdissection



Laser micro dissection



Check and optimize RNA quality and yield

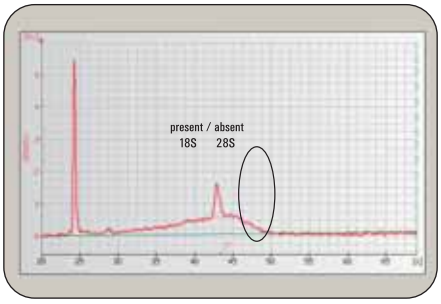
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: RNA derived from laser-microdissected tissue isolated by the PALM®MicroBeam system was shown to be of high quality by convenient analysis with the RNA 6000 Pico assay. RNA-purification kits from different manufacturers and various common staining procedures have been tested and yielded 130-700pg/µl RNA from 1000 cells with different quality (see above). The RNA 6000 Pico kit was well suited to show differences in RNA quality and yield and, therefore, is an ideal tool to optimize and adapt experimental conditions to individual tissue. The experiments were accompanied by a more laborious real time PCR that revealed similar results. Due to its unprecedented sensitivity, the RNA 6000 Pico assay is an indispensable tool for quality control in the context of microdissection experiments, ensuring successful gene expression profiling experiments.

Corresponding application note: 5988-9128EN

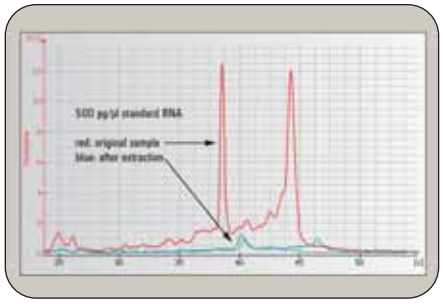
Low amounts of total RNA

Analysis of minimum RNA amounts

A



B



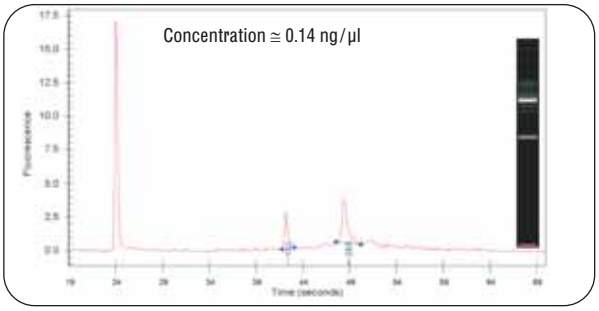
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: The challenge of analysis of minimal amounts of RNA from e.g. laser micro dissections calls for detailed knowledge of extraction conditions. Some commonly used RNA isolation kits and buffer components were assessed in detail. The majority of the kits had no negative effect on the performance of the analysis, whereas, some kits include buffers which lead to shifted, missing and diminished RNA-peaks. In figure A, RNA isolated after microdissection shows lack of the 28S-peak due to high salt concentration introduced during the isolation process. In figure B, a standard RNA was diluted in water and subsequently extracted with a commercially available RNA extraction kit. The original samples (red) and the eluates after extraction are shown. These data show the importance of evaluating the individual method used for RNA extraction to exclude misleading conclusions.

Corresponding application note: 5989-0712EN

Low amounts of total RNA

Genomic DNA in low concentrated RNA extracts

A



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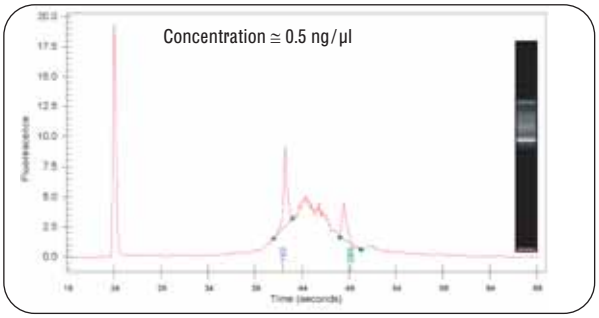
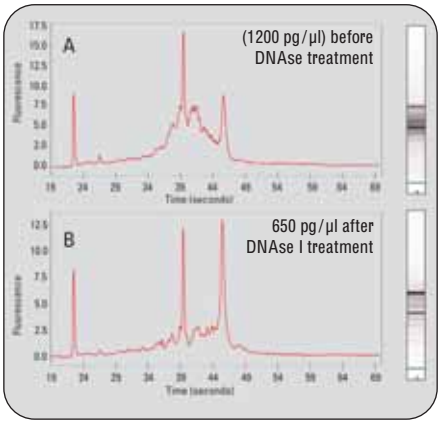


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: Laser capture microdissection enables collection of cells from small tissue areas. A low RNA yield is in the nature of the extraction method from such a specimen that usually complicates quality assessment – a fact that can be circumvented by taking advantage of the Agilent 2100 bioanalyzer capabilities. A comparative study using mouse kidney cryosections showed that on-column DNase digestion is indispensable to obtain a reasonable result for integrity and yield (figure A). Experiments with omitted on-column DNA digestion confirmed that the peak visible in the inter-region consists of genomic DNA which caused overestimation of extracted RNA amounts (figure B).

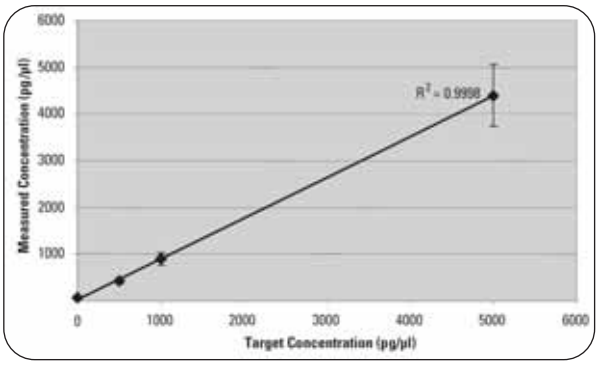
Corresponding application note: 5989-0991EN

Low amounts of total RNA

Low RNA amounts from kidney sections



Renal medulla RNA

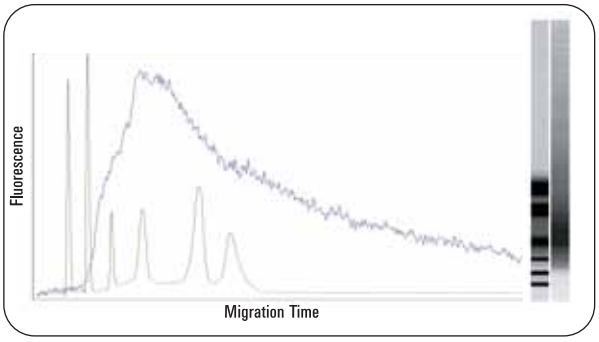


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit are demonstrated for microdissected samples (0.1 mm^3). DNAse I digestion revealed that DNA contamination was present in the sample. Removal of DNA revealed total RNA with a low degree of degradation. Under ideal conditions, the RNA Pico assay has a linear response curve and, therefore, allows estimation of RNA concentrations.

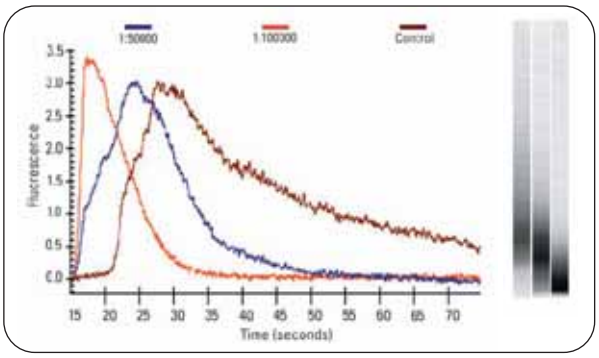
Corresponding application note: 5988-8554EN

Analysis of mRNA

RNA integrity



Highly enriched
Poly (A)+ RNA



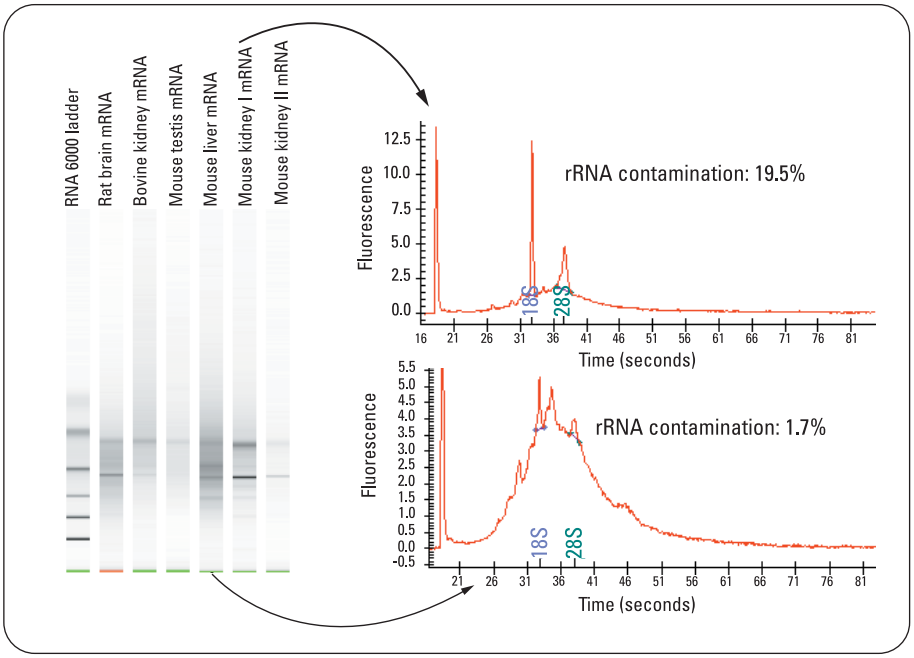
Progressive degradation
of Poly (A)+ RNA

Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Progressive degradation of Poly (A)+ RNA. Poly (A)+ RNA (60 ng/µL) from cultured Jurkat cells was incubated for 15 minutes at room temperature with very dilute RNase A (1 x 10-6 and 2 x 10-6 mg/mL, respectively). A progressive shift towards shorter fragment sizes can be observed. Even with a mild degradation, the absence of very long transcripts can be noticed.

Corresponding application note: 5968-7495EN

Analysis of mRNA

Ribosomal RNA contamination in mRNA samples

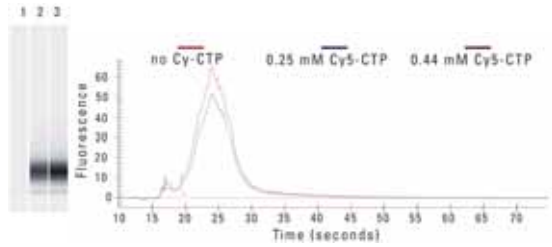


Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Ribosomal contamination in mRNA samples. During the isolation of mRNA, varying amounts of ribosomal RNA can remain in a sample. Since the purity of mRNA is of importance for a number of downstream applications, samples should be checked on the Agilent 2100 bioanalyzer. This slide shows the analysis of 6 commercially available RNA samples from different suppliers. Analysis on the Agilent 2100 bioanalyzer reveals large differences in the purity of the mRNA samples.

Corresponding application note: 5968-7495EN

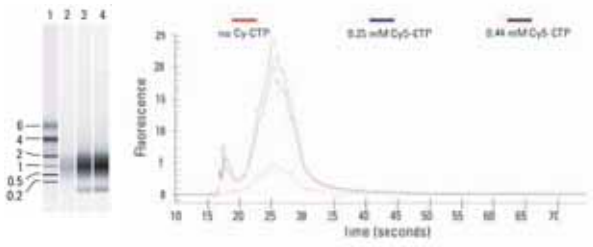
Analysis of Cy5 labeled samples

Analysis of cRNA with and without dye in gel matrix



Assay conditions:
- Cy5 labeled nucleic acids
- no intercalating dye used
- 5nM Cy5 dCTP added to gel matrix and sample buffer for focusing

Lanes:
1. Unlabeled cRNA
2. Cy5 labeled cRNA
3. Cy5 labeled cRNA



Assay conditions:
as above, but intercalating dye included in gel matrix

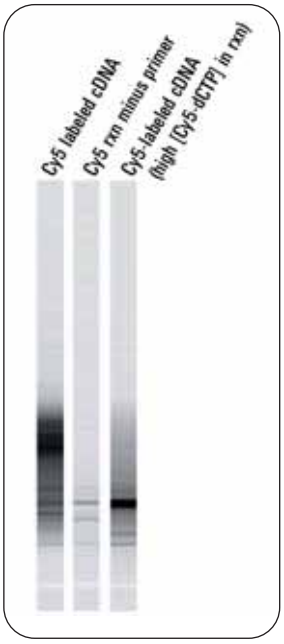
Lanes:
1. RNA transcript ladder
2. Unlabeled cRNA
3. Cy5 labeled cRNA
4. Cy5 labeled cRNA

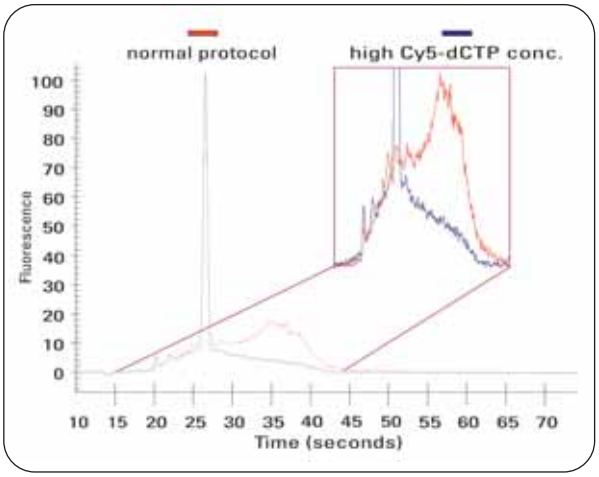
Kit: RNA 6000 Nano kit
Assay: mRNA Nano and Cy5 labeled nucleic acids Nano assay
Application: Analysis of Cy5 labeled and non-labeled cRNA samples. Cy5-labeled samples show the combined signals of the fluorescent label and the RNA signal created by the fluorescence of the RNA 6000 dye. If the RNA 6000 dye is omitted from the gel matrix, only the signal created by Cy5 is detected, allowing the determination of dye incorporation after a labeling reaction. Please note that for Cy3 labeled samples the intactness of the sample can be verified, but the dye incorporation can not be checked.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

Optimization of labeling reactions



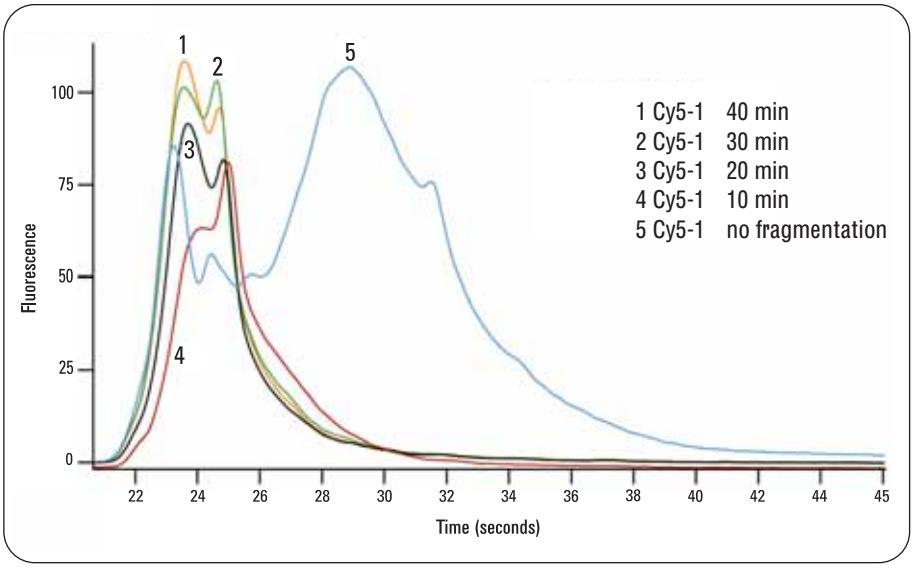


Kit: RNA 6000 Nano kit
Assay: Cy5 Labeled Nucleic Acids Nano assay
Application: An experiment was designed to check the influence of Cy5 dCTP concentration on labeling efficiency. Lane 2 represents the negative control (primer ommitted from the reaction mixture), while lane 3 shows the analysis of a reaction with a 6-fold increased Cy5 dCTP concentration. A look at the electropherograms reveals that the high Cy5 dCTP concentration not only gave a high peak of unincorporated Cy5, but also the labeling efficiency for longer fragments was very low. This approach allows the optimization of labeling reactions.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

cRNA fragmentation

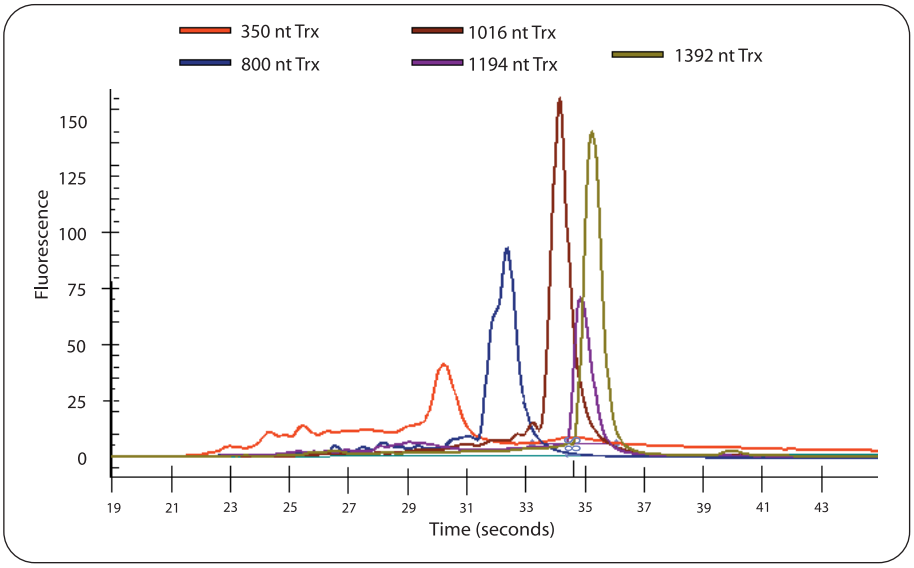


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA 6000 Nano LabChip kit can be used to monitor completion of a cRNA fragmentation reaction. In this example, the profile of a Cy5 labeled cRNA sample was monitored at different time points during a fragmentation reaction. It can be seen that after 10 minutes most of the fragments are in the desired size range. After 20 minutes, no further shift of fragmentation can be observed indicating completion of the fragmentation reaction.

Corresponding application note: 5988-3119EN

Analysis of T7 RNA transcripts

Size estimation



Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: A number of RNA transcripts, ranging from 350 to 1400 nt in size, were analyzed on the RNA 6000 Nano LabChip kit. Although the assay runs under native conditions and the transcripts exhibit a certain degree of secondary structure, a good size estimation can be achieved.

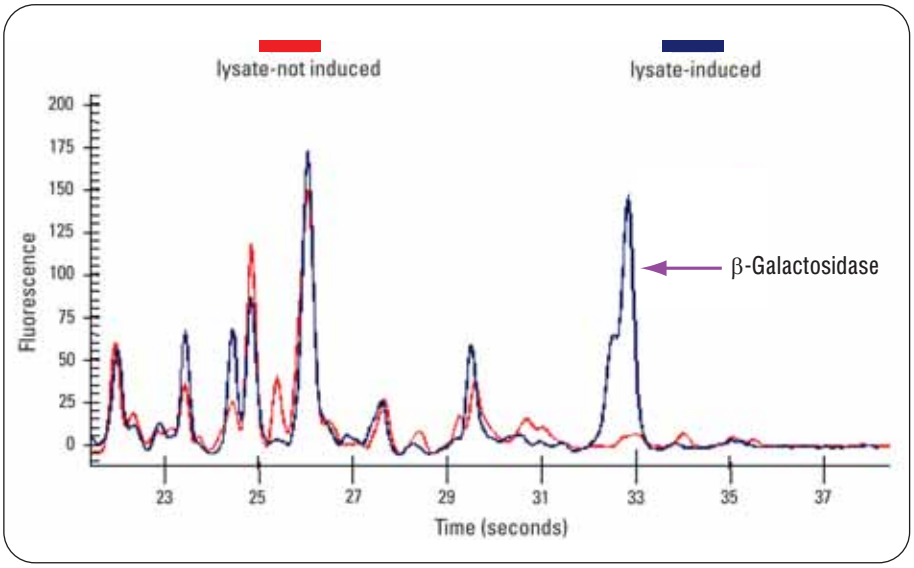
Corresponding application note: data not published

IV. Protein analysis

	Agriculture / Food	Drug Discovery / Drug Development	Drug Manufacturing QA / QC	Genomics	Pharmaceuticals	Proteomics	Forensics / Homeland Security
Protein expression							
Analysis of cell lysates - protein induction			●			●	
Protein purification:							
Comparison between lysate and flow through		●			●		
Analysis of protein purification		●		●		●	
GFP Streptag fusion protein purification		●				●	
Analysis of column capacity						●	
Analysis of column fractions to optimize conditions		●	●			●	
His-tag protein purification with Ni++ ZipTips®		●	●			●	
Enzymatic removal of His-tags from recombinant proteins			●			●	
Complementing RP-HPLC protein purification		●			●	●	
Antibody analysis							
Analysis of antibodies under reducing and non-reducing conditions		●	●				
Quantitation of the half-antibody content in IgG_4 preparations		●	●		●		
Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis		●	●			●	
Absolute quantitation of IgG		●	●			●	
Quality control of stressed antibodies		●	●				
Separation of bispecific antibodies chains		●	●		●	●	
Food analysis							
Bovine milk analysis	●					●	
Protein pattern of different transgenic seedlines	●					●	
Protein - others							
Absolute protein quantitation	●		●		●	●	
Glycoprotein sizing	●		●			●	
Protein quality control prior to MS-analysis			●			●	
Depletion of high abundant proteins from blood samples		●			●		
Increased sensitivity by desalting protein samples	●	●			●	●	

Protein expression

Analysis of cell lysates - protein induction

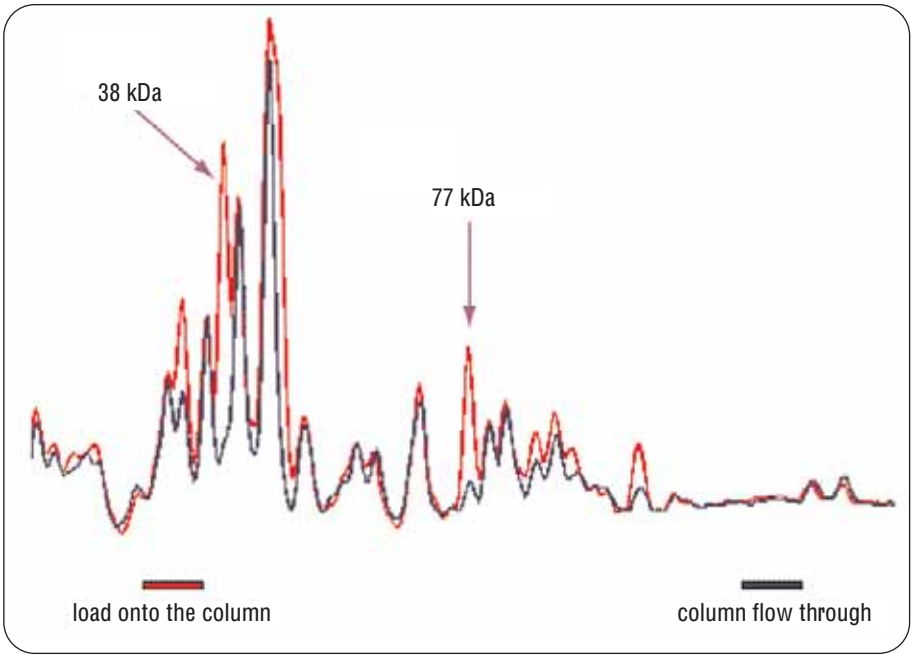


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Two cell lysates, induced and non-induced were compared to verify the induction of protein expression. The overlay feature of the bioanalyzer software allows quick sample comparison. The blue electropherogram trace shows the cell lysate highly expressing β-galactosidase (128 kDa).

Corresponding application note: data not published

Protein purification

Comparison between lysate and flow through

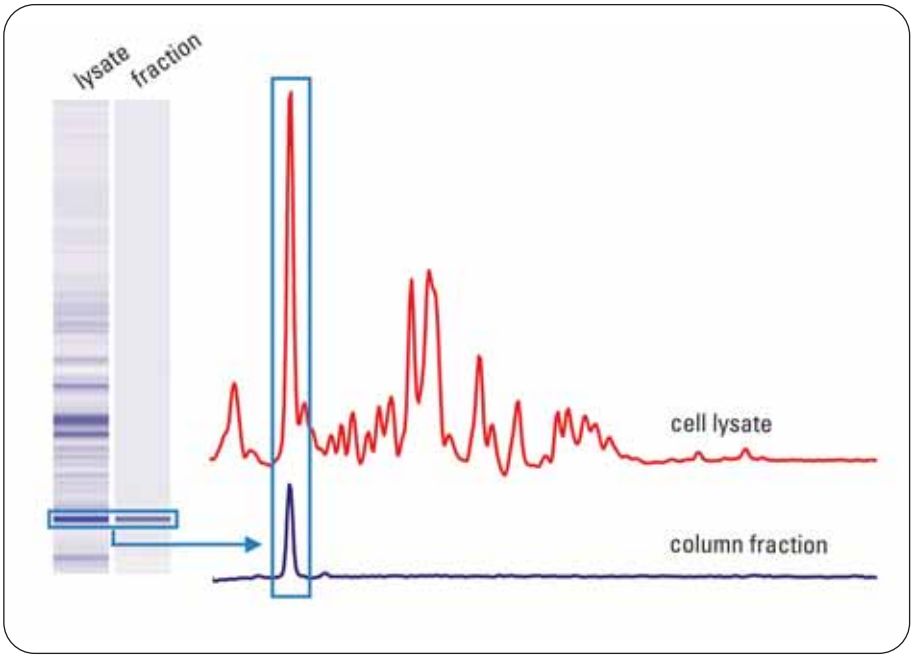


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Cells were lysed using the Pierce B-Per kit and then loaded onto an affinity column. The protein of interest, a 38 kDa protein, should bind to the column and not show up in the flow through. By overlaying the 2 electropherograms from both samples, the lysate and the flow through, it is visible that the protein of interest has bound to the column as expected. In addition, a 77 kDa protein has bound to the column, which could be attributed to unspecific binding or the binding of a dimer.

Corresponding application note: data not published

Protein purification

Analysis of protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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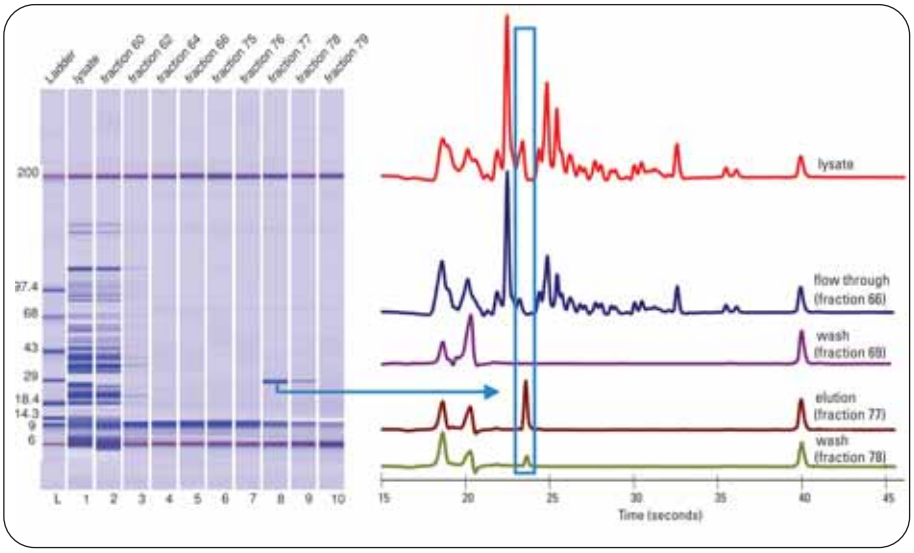
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A 18 kDa protein was purified using affinitiy chromatography. The starting material and the column fraction were analyzed with the protein assay. The protein of interest was determined to be 99% pure and the concentration in the column fraction was 167 ng/µl.
The protein assay allows protein purity and concentration to be determined in one step, in addition it calculates protein size for reconfirmation.

Corresponding application note: data not published

Protein purification

GFP Streptag fusion protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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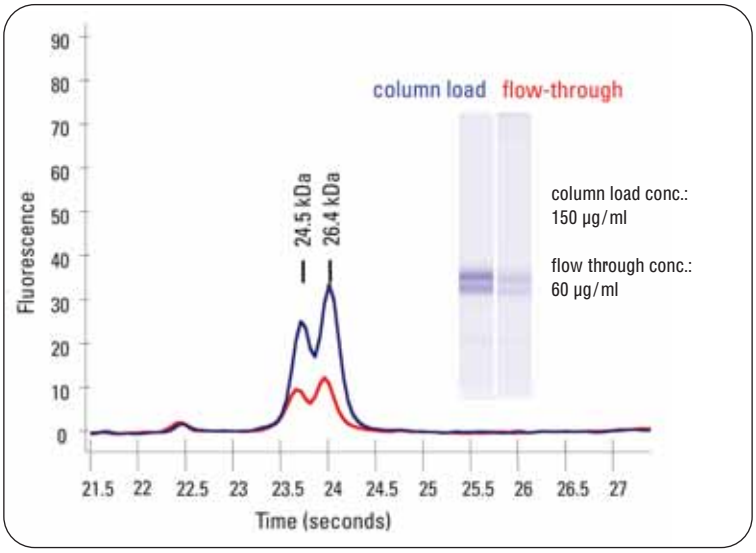
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: This example shows the analysis of various steps during the purification workflow of a GFP Streptag fusion protein (28 kDa). The protein was expressed in *E.coli* and purified via affinity chromatography with Strep Tactin Poros as the column matrix. The protein assay allows each purification step from the cell lysis to the elution of the purified protein to be monitored and optimized.

Corresponding application note: 5988-5025EN

Protein purification

Analysis of column capacity

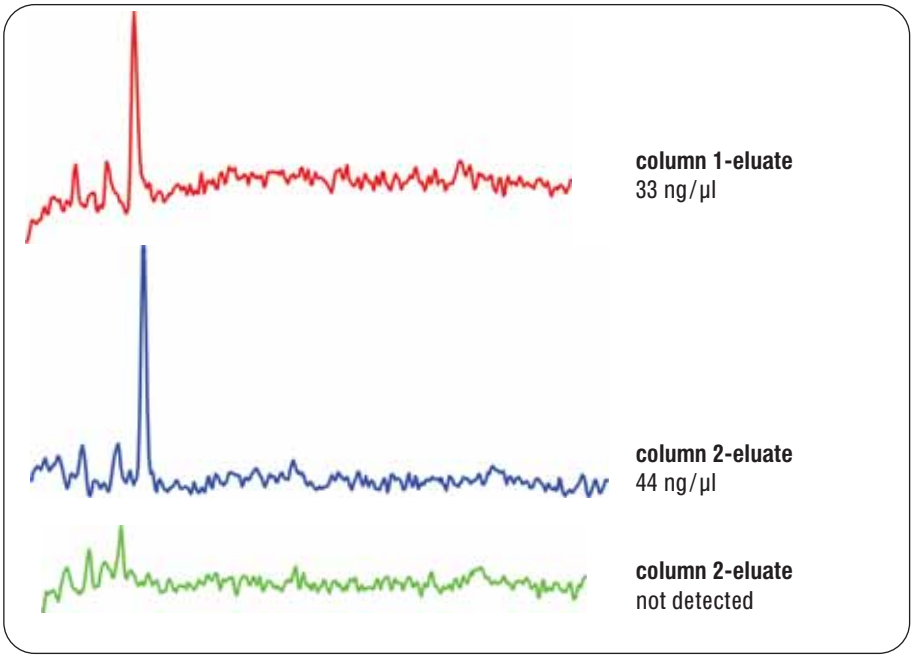


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The binding of a recombinant antibody Fab fragment to a Sepharose column with immobilized Protein G was analyzed to determine the column capacity and prevent column overloading. The protein assay allows this purification step to be monitored and quickly optimized.

Corresponding application note: 5988-4022EN

Protein purification

Analysis of column fractions to optimize conditions



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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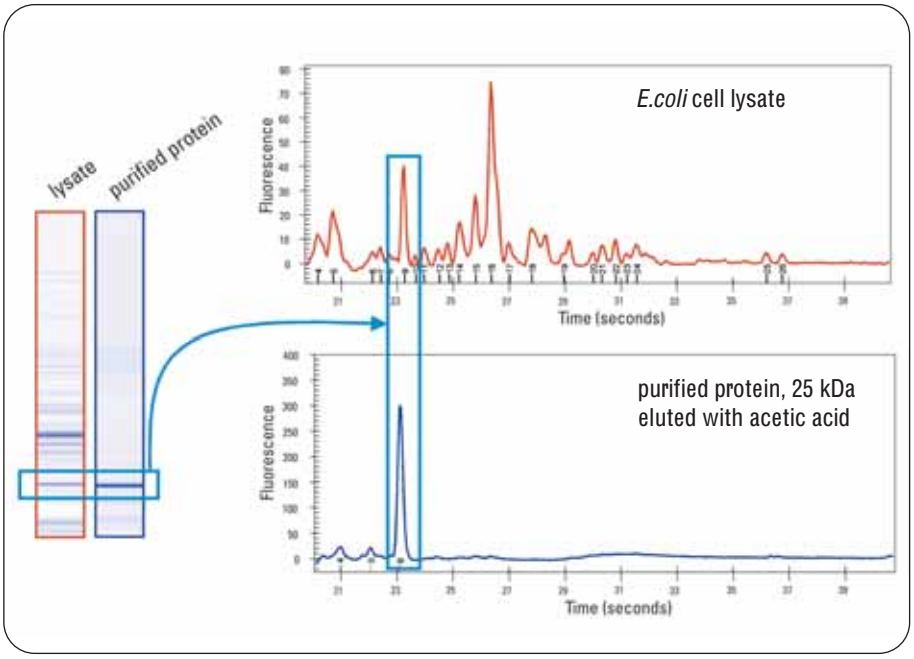
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Different column conditions were tested to optimize the purification conditions for a 30 kDa protein. The column fractions were analyzed for protein purity and concentration to identify the optimal conditions providing a highly purified protein in a good yield. Using the protein assay it was possible to determine the optimum purification conditions in a short time frame.

Corresponding application note: data not published

Protein purification

His-tag protein purification using Ni++ZipTips®

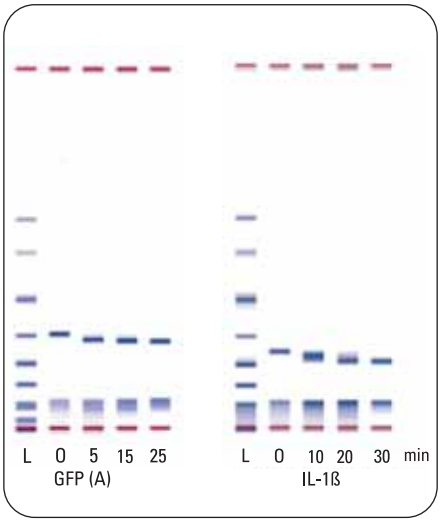


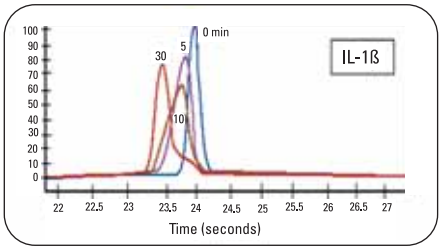
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: ZipTips loaded with a Ni^{2+}-resin (in development by Millipore) were used to purify a His-tagged protein expressed in *E.coli*. Both the cell lysate and the purified protein were analyzed with the Agilent 2100 bioanalyzer to demonstrate the performance of the tips.
The purification with the tips takes approximately 5 minutes, usually followed by the analysis of the samples with SDS-PAGE analysis which takes a further 2 hours. The SDS-PAGE analysis was substituted by the much faster Protein 200 Plus assay run on the Agilent 2100 bioanalyzer.

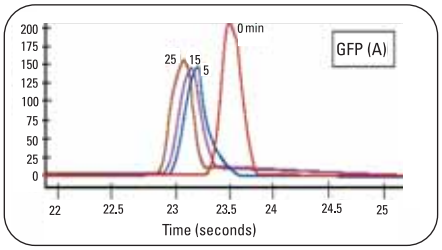
Corresponding application note: data not published

Protein purification

Enzymatic removal of His-tags from recombinant proteins







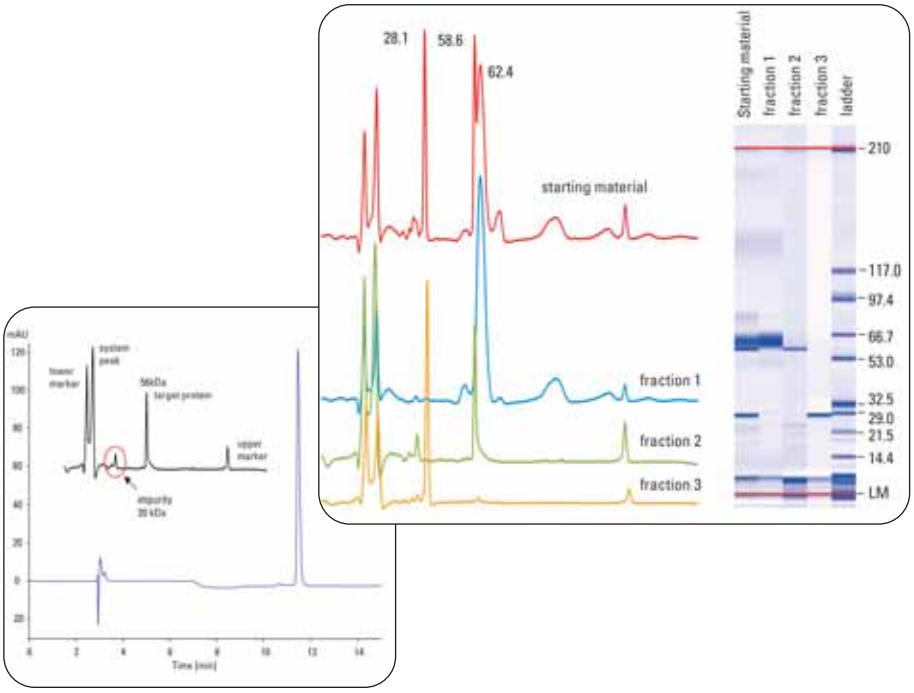
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: For some applications, it might be necessary to remove the His-tag after the protein purification because of its effects on enzymatic activity or protein structure. Here the TAGZyme system (Qiagen) was used to remove the N-terminal His-tag from two different proteins, a GFP variant and a recombinant Interleukin 1β. Samples were taken at different time points to study the kinetics of the enzymatic cleavage. The dipeptide cleavage can be detected by a size shift on the gel-like images and the electropherograms. The fast analysis with the bioanalyzer allows multiple kinetic studies to done in one day instead of waiting until the next day for the results from SDS-PAGE analysis.

Poster presented at ABRF Conference, March 2002 by
F. Schäfer, K. Steinert, C. Feckler, J.Drees, and J.Ribbe, QIAGEN GmbH, Hilden, Germany

Corresponding application note: 5988-8144 EN

Protein purification

Complementing RP-HPLC protein purification

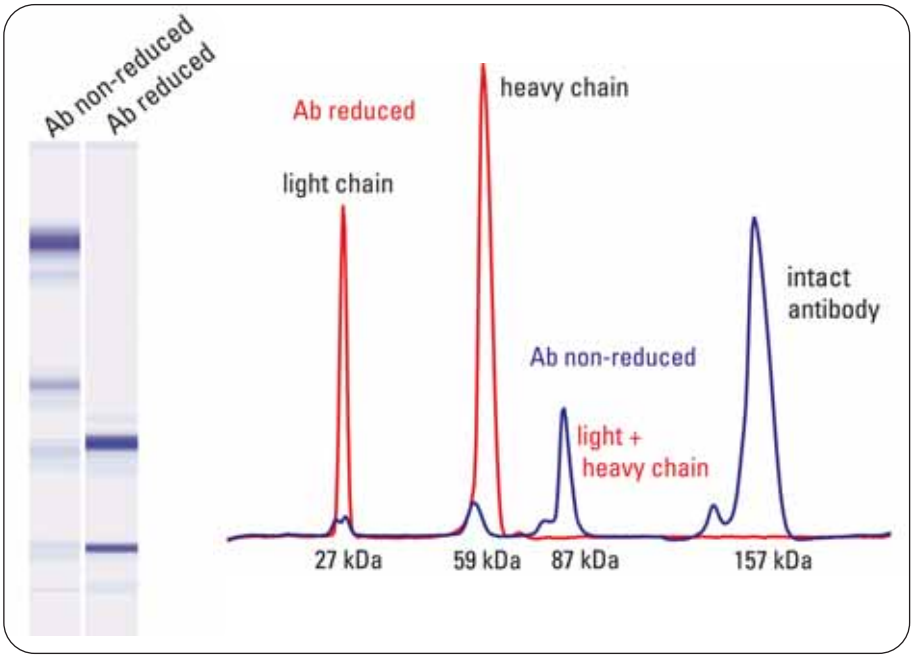


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification and characterization was carried out facilitating an Agilent 1100 Series purification system for reverse phase HPLC assisted by the Agilent 2100 bioanalyzer. The final polishing of a 56 kDa protein by RP HPLC from a pre-purified sample (starting material, right: red electropherogram and gel) and the analysis of three HPLC-fractions containing the major components are shown (fractions 1-3). No impurity is visible by RP HPLC reanalysis (left chromatogram, fraction 2) of the fraction containing the target protein. However, because the Agilent 2100 bioanalyzer is an orthogonal technique compared to reverse phase HPLC a 20 kDa protein could be found as an impurity (see insert). The reverse phase HPLC purification leads to a purity of only 76% for the protein of interest and the Agilent 2100 bioanalyzer reveals the necessity of further purification.

Corresponding application note: 5988-8630EN

Antibody analysis

Analysis of antibodies under reducing and non-reducing conditions

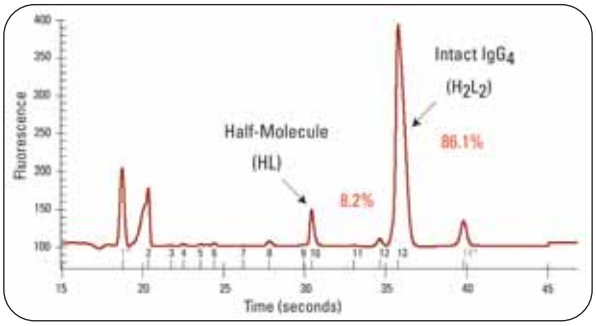


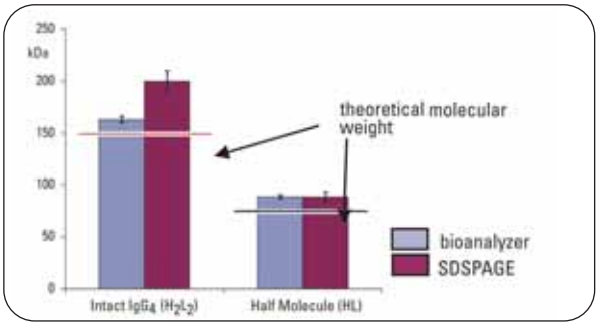
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The protein kit allows analysis of both reduced and non-reduced antibodies on the same chip. This is not possible using SDS-PAGE, as the reducing agent will diffuse within the gel and will also reduce other samples. Under non-reducing conditions, it is expected to detect the intact antibody around 160 kDa. Here the single light and heavy chains and half-antibodies are also visible. Under reducing conditions this is all completely reverted to single light and heavy chains, due to the reduction of the disulfide bonds.

Corresponding application note: data not published

Antibody analysis

Quantitation of the half-antibody content in IgG_4 preparations





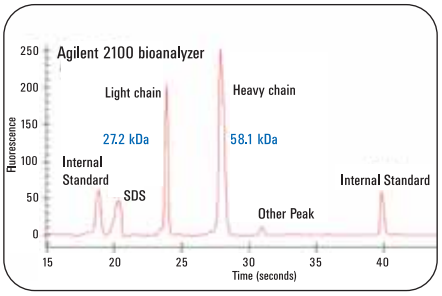
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In the given host cell line for antibody production usually up to 30% of IgG_4 is secreted as half molecule (half antibody). The half-molecule has only a single disulfide bond between the heavy and light chains, the inter-heavy chain disulfide bonds are absent. The protein assay allows the half-antibody content in IgG_4 preparations to be determined automatically.
In addition, the sizing provided by the Agilent 2100 bioanalyzer compares very well to the theoretical size and is superior to SDS-PAGE in terms of accuracy and reproducibility.

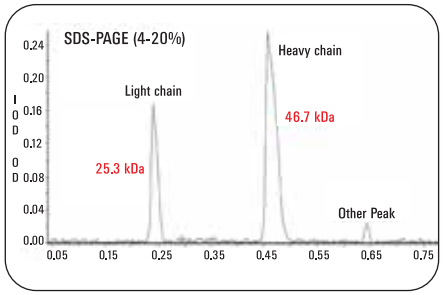
Poster presented at WCBP Conference, January 27-30, 2002 by E. Vasilyeva, H. Fajardo, P. Bove, F. Brown and M. Kretschmer. BIOGEN, Cambridge, MA , USA

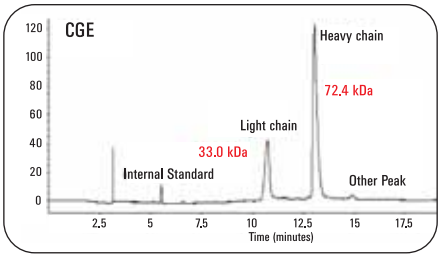
Corresponding application note: data not published

Antibody analysis

Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis







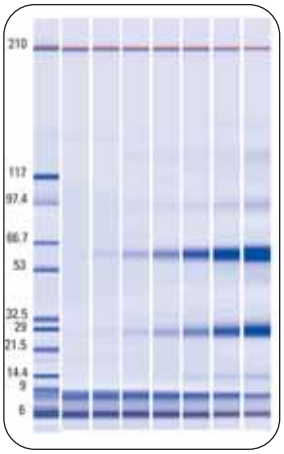
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The analysis of a humanized monoclonal antibody under reducing condition was compared using 3 different techniques, the Agilent 2100 bioanalyzer, 4-20% SDS-PAGE, stained with Coomassie, and capillary gel electrophoresis. All 3 techniques result in a similar separation pattern showing the light and the heavy chain of the antibody. In addition, the determined sizes of the light and heavy chain were comparable for all 3 techniques and compared well to the molecular weights determined by MALDI-TOF (light chain: 23762 Da, heavy chain: 51003 Da). However, the Agilent 2100 bioanalyzer provides significant time saving compared to the other techniques.

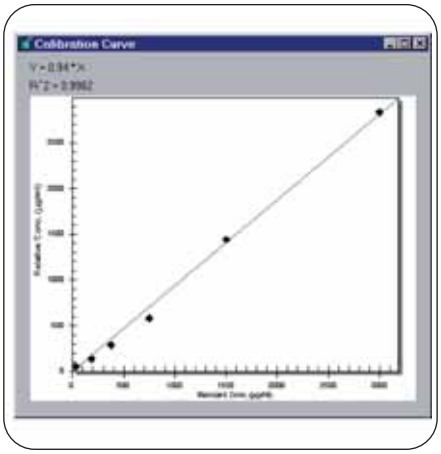
Poster presented at WCBP Conference, January 2002 by
S.H. Bowen, M. Chan, P. McGeehan, J. Smith, L. Inderdass, R. Strouse, M. Schenerman
MedImmune Inc., Gaithersburg, MD, USA

Corresponding application note: data not published

Antibody analysis

Absolute quantitation of IgG



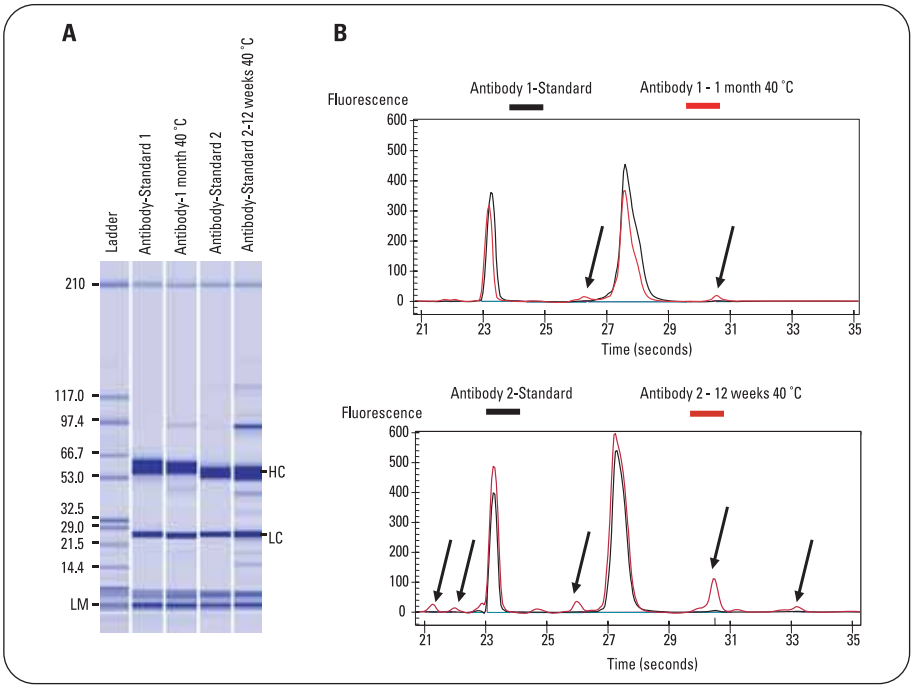


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The calibration feature of the software allows determination of the absolute antibody concentration in comparison to user defined standards with known concentration, accurate determination of IgG concentrations and carrying out batch comparison during antibody QA/QC.

Corresponding application notes: 5988-4021EN and 5988-6576EN

Antibody analysis

Quality control of stressed antibodies

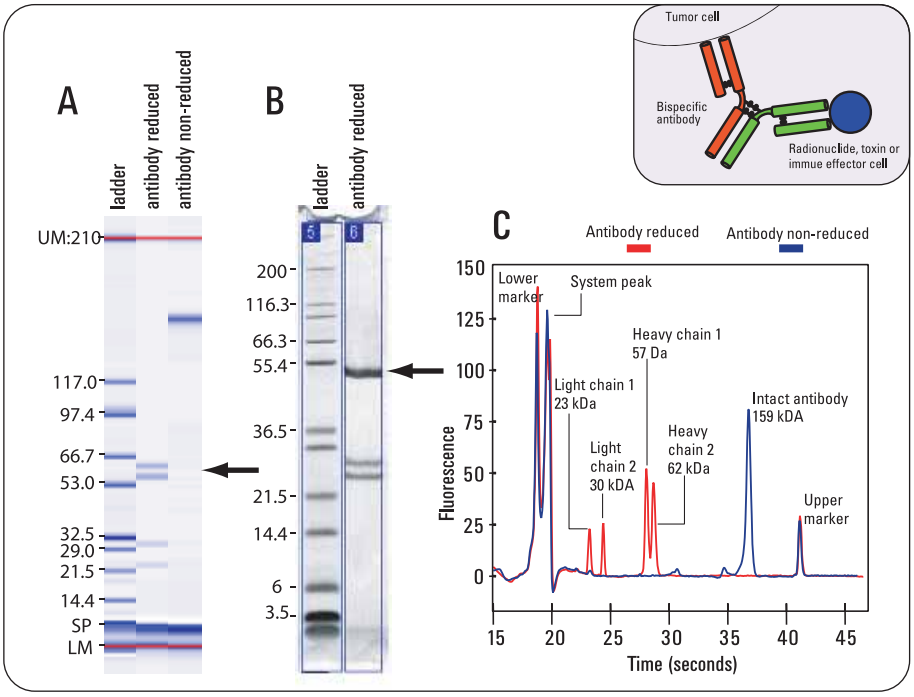


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A quality control step in pharmaceutical QA/QC departments is to trigger typical degradation and aggregation patterns for a specific antibody. The given samples from heat stress stability studies show expected protein byproducts after aging at elevated temperatures. The content of heavy and light chain, representing the intact antibody, is reduced by 5% or 13% within 1 month or respectively 12 weeks. Excellent reproducibility in the range from 0.6 to 1.7% CV for this quantification was achieved in a validation study with three different users and two bioanalyzer instruments over several days.

Corresponding application note: 5988-9648EN

Antibody analysis

Separation of bispecific antibodies chains

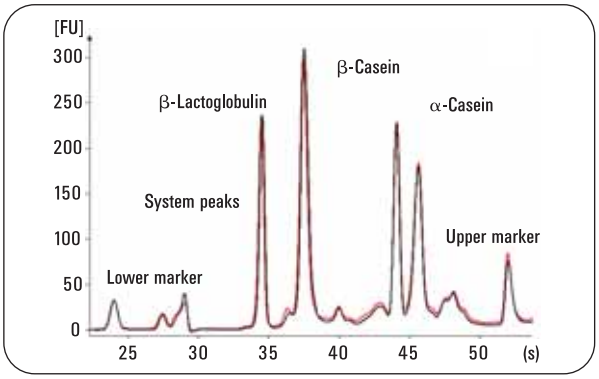


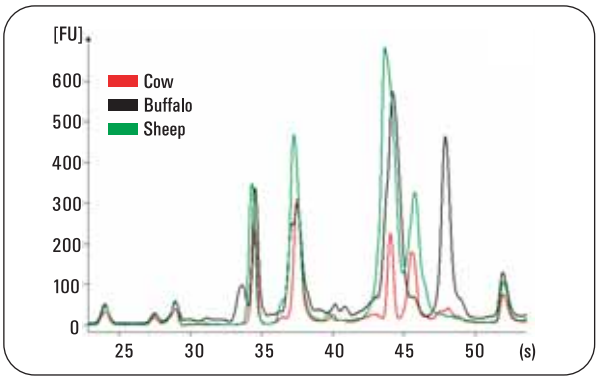
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In general, antibodies are biopharmaceuticals of great interest. Especially bispecific antibodies often require high resolution to allow analysis of both sets of chains (Agilent 2100 bioanalyzer: A, gel like view, resolved heavy chains; C electropherogram). A labor intensive SDS-PAGE could not resolve the heavy chains (B, marked by an arrow) in the given sample. In contrast, the Agilent 2100 bioanalyzer is a superior tool for antibody quality control since it is a convenient, fast and easy to standardize method which additionally enables quantitative analysis.

Corresponding application note: 5988-9651EN

Food analysis

Bovine milk analysis



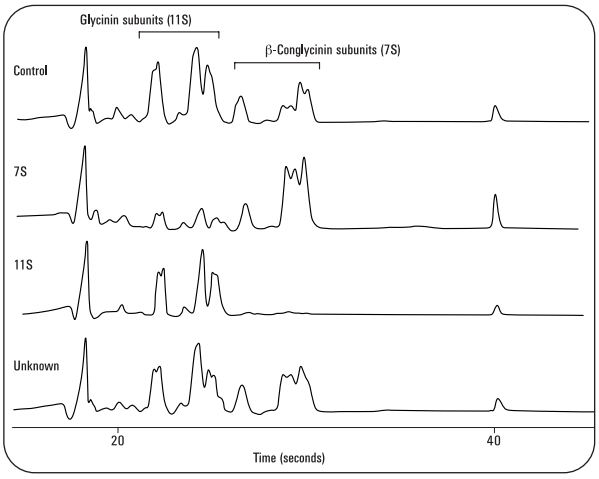


Kit: Protein 50 kit
Assay: Protein 50 assay
Application: The Protein 50 LabChip is suitable for analysis of diary products such as milk. The kit delivers an excellent reproducibility, as shown in this example (A, bovine milk diluted 1:10). Here the main protein fractions can be identified running the individual purified proteins for comparison (not shown). The overlay of the electropherograms from two separate runs under reducing conditions demonstrates the high reproducibility of the assay. Furthermore, milk from different animals could be distinguished based on their protein pattern (B) which facilitates a fast incoming inspection in routine labs.

Corresponding application note: data not published

Food analysis

Protein pattern of different transgenic seedlines



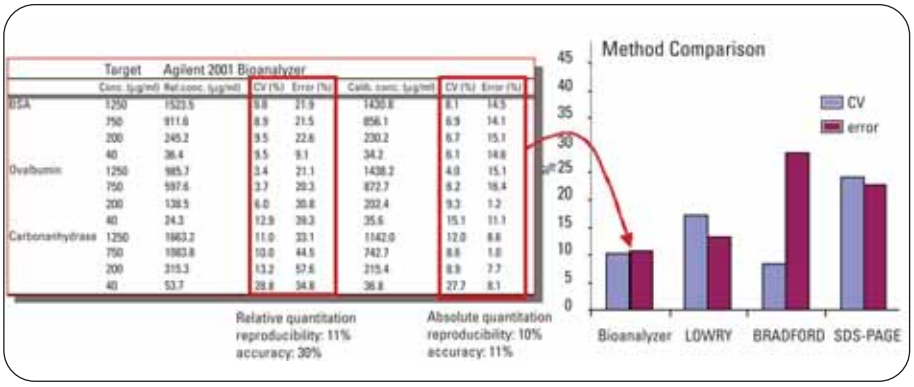
SeedLine	Extracted protein level µg/ml	7S/11S Ratio
Control	14,000	0.39±0.004(n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknows	13,000	0.72±0.1(n=20)

Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Determination of protein size and concentration with sufficient accuracy and precision allows the highly efficient characterization of transgenic seed lines. Expressed protein was available after grinding, extraction of seeds and dilution with buffer. Electropherograms were evaluated by integration of regions specific for 7S or 11S seed storage proteins. The elevated ratio of 7S/11S for the analyzed unknown line shows significant changes in the expression profile in comparison with the control.

Corresponding application note: 5988-9441EN

Protein - others

Absolute protein quantitation

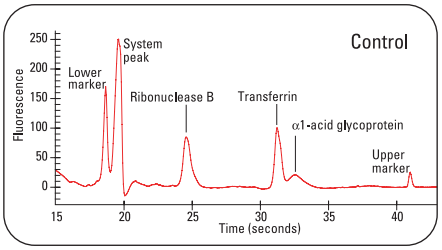


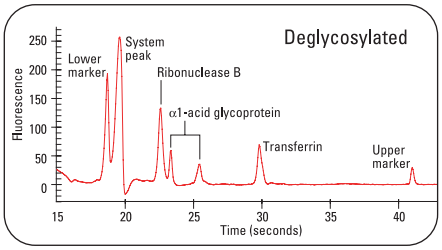
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A comparative analysis of different techniques used for absolute protein quantitation was performed analyzing 3 different proteins (CA, BSA, OV) in 4 different concentrations (40 - 1250 ug/ml). The same samples were quantitated using the Agilent 2100 bioanalyzer, two commonly used total protein quantitation assays, Lowry and Bradford, and SDS-PAGE, stained with Coomassie. The relative standard deviation (CV) and the error compared to the target concentration were determined. A comparison shows that the CV and error for the Agilent 2100 bioanalyzer are better than for the SDS-PAGE by a factor of 2. This data demonstrates that the Agilent 2100 bioanalyzer is a viable alternative for protein quantitation. It allows the quantitation of individual proteins and simultanous determination of protein purity and size.

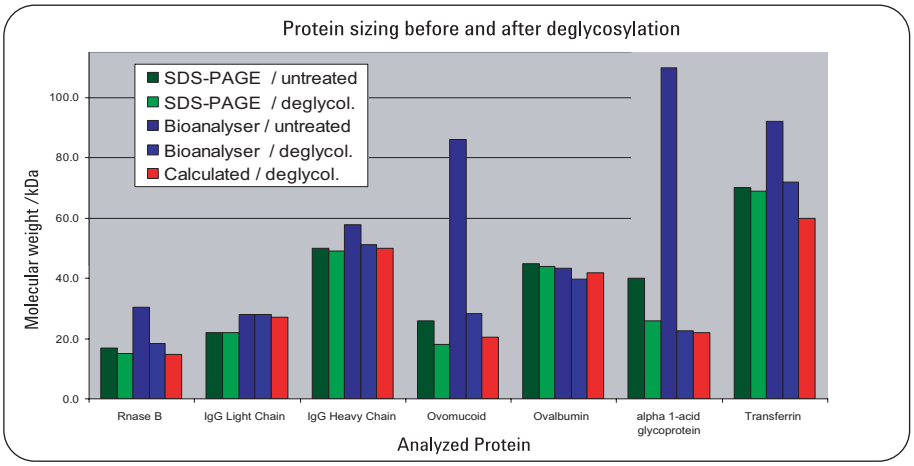
Corresponding application notes: 5988-4021EN and 5988-6576EN

Protein - others

Glycoprotein sizing





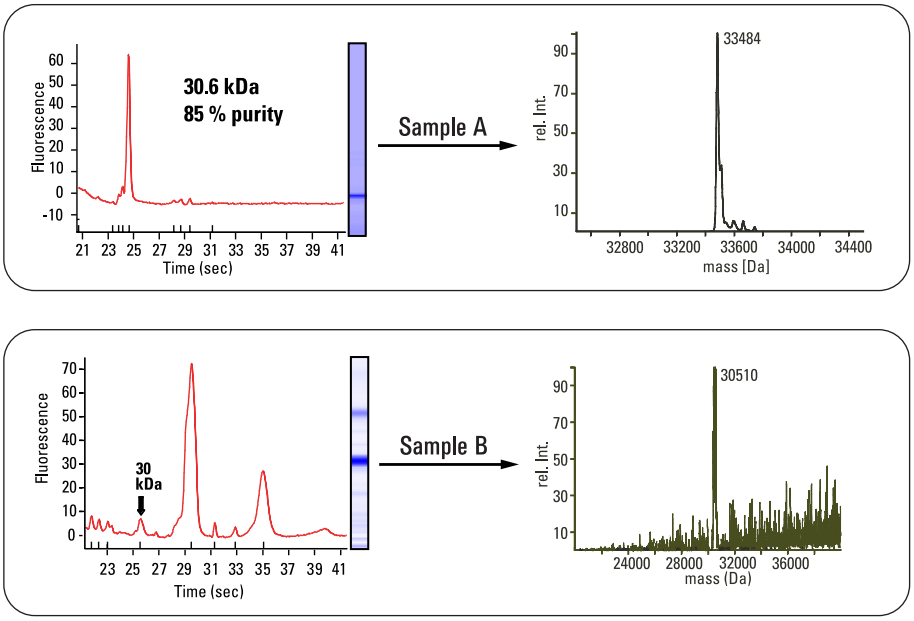


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Due to large carbohydrate moieties glycosylated proteins can differ in amount of incorporated SDS and shape of the protein/SDS-complex from non-glycosyslated proteins. This may lead to different migration times in SDS-PAGE, as well as in the Protein 200 Plus assay run on the Agilent 2100 bioanalyzer. The data compare deglycosylation of a mixture of three proteins (electropherogram on the left) with a commercial N-Glycosidase F Deglycosylation kit. Sizing experiments comparing glycosylated and non glycosylated states for additional proteins are compared and summarized on the right. Such an approach avoids misinterpretation of sizing due to glycosylation and allows detection of a posttranslational modification of unknown proteins.

Corresponding application note: 5989-0332EN

Protein - others

Protein quality control prior to MS-analysis

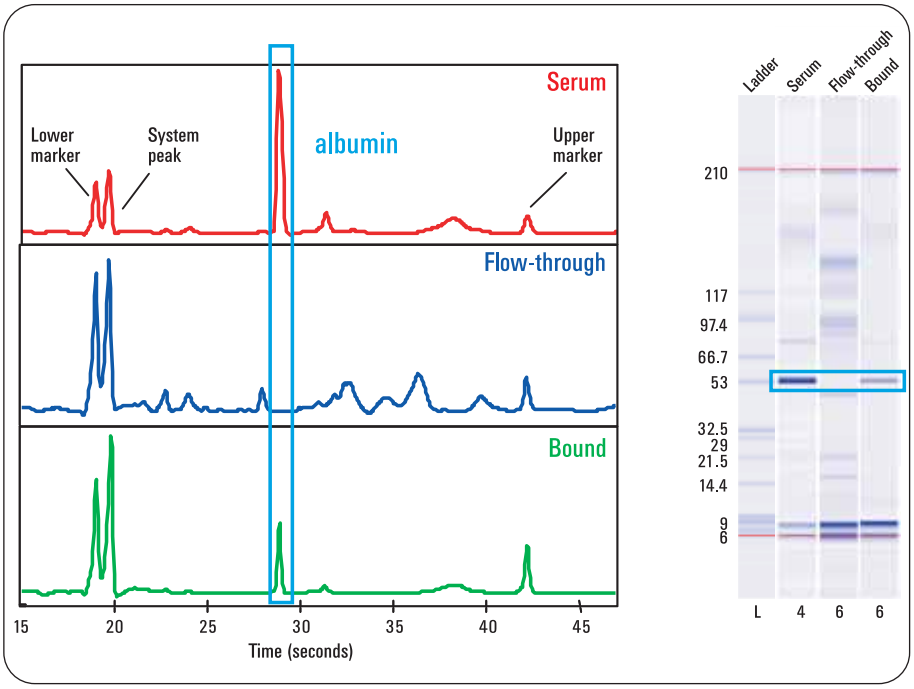


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: By applying soft ionization methods like MALDI or ESI mass spectroscopy (MS), mass information from proteins up to 300kDa can be obtained. However, proper sample preparation is an important precondition. Concentration, purity and assumed size are valuable ex ante information usually given by biochemists to MS-analysis services. Two different examples for proteins analyzed by an LC/MS-method (right panel) with good results for sample A and discrepancies for sample B are shown. An analysis of the samples with the Protein 200 Plus assay (left panel) showed an impure protein preparation for sample B. Here, two major peaks at higher masses (66 and 132kDa) potentially caused by aggregates were encountered. The protein of interest (30kDa) yielded high noise background in the MS. A quality check of the sample with the Protein 200 Plus assay, therefore, may avoid an unproductive MS analysis or data evaluation.

Corresponding application note: 5989-0771EN

Protein - others

Depletion of high abundant proteins from blood samples

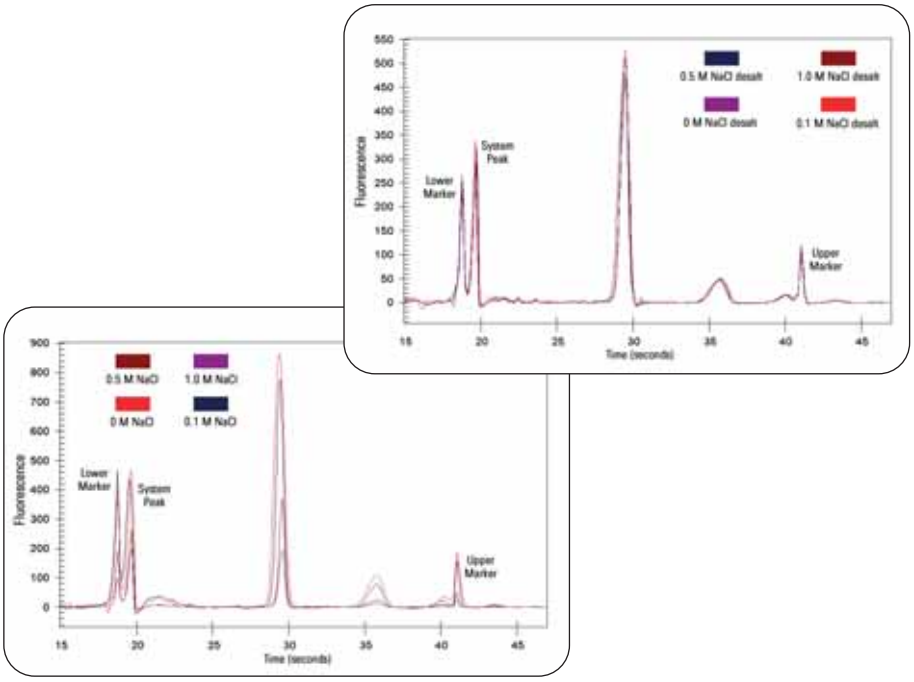


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Depletion of high abundant proteins in human blood plasma was facilitated by the Agilent Multiple Affinity Removal System. Unprocessed serum, the flow through (i.e. the immunodepleted serum) and the bound proteins after specific elution were analyzed on the Agilent 2100 bioanalyzer. Equivalent amounts were analyzed and resulting electropherograms (left) and gel like images (right) show in comparison the completeness of separation. The Agilent 2100 bioanalyzer, in combination with the Protein 200 Plus LabChip kit, proves to be an excellent method for evaluation of serum processed with albumin removal kits. The system offers a rapid and accurate method to detect proteins both quantitatively and qualitatively.

Corresponding application note: data not published

Protein - others

Increased sensitivity by desalting protein samples



Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification steps, such as ion exchange chromatography, often implicate high salt concentrations. Nevertheless, quantitation of these samples is effective since the upper marker serves as internal protein standard and is subjected to the same conditions. However, under high salt conditions lower amounts of protein are injected into the microfluidic channels for analysis. Therefore, the sensitivity can be increased by usage of convenient desalting spin columns. Comparably higher and homogeneous peak intensities are obtained while the recovery after such treatment is good.

Corresponding application note: 5989-0228EN

Literature

Microfluidics application notes from Agilent Technologies

To download an application note visit the library section at:
www.agilent.com/chem/labonachip

Description	Publication number
DNA	
Quantitative analysis of PCR fragments with DNA 7500 LabChip kit	5968-7496EN
High precision restriction fragment sizing with DNA 12000 LabChip kit	5968-7501EN
Comparing the Agilent 2100 bioanalyzer performance to traditional DNA analysis	5980-0549EN
Agilent 2100 bioanalyzer replaces gel electrophoresis in prostate cancer research	5988-1086EN
High resolution DNA analysis with the DNA 500 and DNA 1000 LabChip kits	5988-3041EN
Quantitative end-point RT-PCR gene expression using DNA 7500 LabChip kit	5988-3674EN
Development of meat speciation assays using the Agilent 2100 bioanalyzer	5988-4069EN
Analysis of genetically modified soya using the Agilent 2100 bioanalyzer	5988-4070EN
Detecting genetically modified organisms with the Agilent 2100 bioanalyzer	5988-4847EN
Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method	5988-9341EN
Highly efficient multiplex PCR using novel reaction chemistry	5988-9342EN
Microfluidic analysis of multiplex PCR products for the genotyping of Helicobacter pylori	5989-0078EN
Nested multiplex PCR for the determination of DNA from genetically modified corn and soy beans using the Agilent 2100 bioanalyzer	5989-0124EN
Rapid detection of genomic duplications and deletions using the Agilent 2100 bioanalyzer	5989-0192EN
Mutation detection for the K-ras and P16 genes	5989-0487EN
Use of the Agilent 2100 bioanalyzer and the DNA 500 LabChip in the analysis of PCR amplified mitochondrial DNA	5989-0985EN
Assessing genomic DNA contaminations of total RNA isolated from kidney cells obtained by Laser Capture Microdissection using the Agilent RNA 6000 Pico assay	5989-0991EN
Integrating high-throughput, on-chip electrophoresis analysis into PCR diagnostics projects	5989-1870EN
DNA QC for Oligonucleotide Array CGH (aCGH) with the Agilent 2100 bioanalyzer	5989-2487EN
Label-free analysis of microsatellite instability in colorectal carcinoma by on-chip electrophoresis	5989-2626EN
Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 bioanalyzer	5989-2982EN
Using the Agilent 2100 bioanalyzer to Optimize the PCR Amplification of Mitochondrial DNA Sequences	5989-3107EN
Analysis of Cy5-labeled cRNAs and cDNAs using the Agilent 2100 bioanalyzer and the RNA 6000 LabChip kit	5980-0321EN
Measuring the METH-2 promoter hypermethylation and transcript dwonregulation in non-small cell lung carcinomas with the Agilent 2100 bioanalyzer	5989-3514EN

RNA

Analysis of total RNA using the RNA 6000 LabChip kit	5968-7493EN
Analysis of messenger RNA using the RNA 6000 LabChip kit	5968-7495EN
Analysis of Cy5-labeled cRNAs and cDNAa using the RNA 6000 LabChip kit	5980-0321EN
Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis and UV spectrometry	5988-7650EN
Characterization of RNA quality using the RNA 6000 LabChip kit	5980-0472EN
Comparing performance of the Agilent 2100 bioanalyzer to traditional RNA analysis	5980-2206EN
The total RNA story	5988-2281EN
Interpreting mRNA electropherograms	5988-3001EN
Optimizing cRNA fragmentation for microarray experiments using Agilent 2100 bioanalyzer	5988-3119EN
High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit	5988-8554EN
Quality assurance of RNA derived from laser microdissected tissue samples obtained by the PALM MicroBeam system using the RNA 6000 Pico LabChip kit	5988-9128EN
Advancing the quality control methodology to assess isolated total RNA and generated fragmented cRNA	5988-9861EN
siRNA transfection optimization with the Agilent 2100 bioanalyzer	5988-9872EN
Successful analysis of low RNA concentrations with the Agilent 2100 bioanalyzer and the RNA 6000 Pico	5989-0712EN
Stringent RNA quality control using the Agilent 2100 bioanalyzer	5989-1086EN
Gene Expression Profiling of Esophageal Cancer Using Laser Capture Microdissected Samples	5989-1088EN
RNA Integrity Number (RIN) - Standardization of RNA Quality Control	5989-1165EN
Confirming gene silencing mechanism by pGFP/GFP22 - siRNA co-transfection	5989-0103EN
Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method	5988-9341EN
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High-Purity RNA isolation from a wide range of plant species and tissue types	5989-2271EN
Isolation of high purity total cellular RNA from muscle tissues	5989-2312EN

Cells

Apoptosis detection by annexin V and active caspase-3 with the Agilent 2100 bioanalyzer	5988-4319EN
Detection of cell surface proteins with the Agilent 2100 bioanalyzer by on-chip antibody staining	5988-7111EN
Monitoring transfection efficiency in cells using an on-chip staining protocol	5988-7296EN
A fast protocol for apoptosis detection by Annexin V with the Agilent 2100 bioanalyzer	5988-7297EN
Cell fluorescence assays on the Agilent 2100 bioanalyzer - general use	5988-4323EN
Monitoring transfection efficienency by green fluorescence protein (GFP) detection	5988-4320EN
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Measuring multiple apoptosis parameters with the Agilent 2100 bioanalyzer	5988-8028EN
Flow cytometric analysis of human primary cells using the Agilent 2100 bioanalyzer and on-chip staining	5988-8154EN
Confirming gene silencing mechanism by pGFP/GFP22 - siRNA co-transfection	5989-0103EN
Flow cytometric analysis of a limited number of cells using the Agilent 2100 bioanalyzer	5989-0746EN
A new method for the calculation of baculovirus titre using the Agilent 2100 bioanalyzer and the flow cytometry kit	5989-1644EN
Cytometric analysis of Upregulated Functional Gene Expression in Primary Cells by On-Chip Staining	5989-2718EN
Detection of Apoptosis in Primary Cells by Annexin V Binding Using the Agilent 2100 bioanalyzer	5989-2934EN
siRNA transfection optimization with the Agilent 2100 bioanalyzer	5988-9872EN

Proteins

Protein sizing and analysis using the Protein 200 LabChip kit	5988-0975EN
Differences and similarities between Protein 200 Assay and SDS-PAGE (technical note)	5988-3160EN
Comparison of different protein quantitation methods	5988-6576EN
Using the Agilent 2100 bioanalyzer for analysis of His-tag removal from recombinant proteins	5988-8144EN
Absolute quantitation with the Protein 200 LabChip kit	5988-4021EN
Optimization of protein purification using the Agilent 2100 bioanalyzer	5988-4022EN
Comparison of different methods for purification analysis of a green fluorescent strep-tag fusion protein	5988-5025EN
Fast analysis of proteins between 5-50 kDA using the Agilent 2100 bioanalyzer and Protein 50 assay	5988-8322EN
Using the Agilent 2100 bioanalyzer for analysis of His-tag removal from recombinant proteins	5988-8144EN
Protein purification and characterization using the 1100 Series purification system and the Agilent 2100 bioanalyzer	5988-8630EN
Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 bioanalyzer	5988-9441EN
Quality control of antibodies using the Agilent 2100 bioanalyzer and the Protein 200 Plus assay	5988-9648EN
Analysis of bispecific antibodies using the Agilent 2100 bioanalyzer and the Protein 200 Plus assay	5988-9651EN
Evaluation of albumin removal using the Agilent 2100 bioanalyzer	5988-9911EN
Increased sensitivity by desalting protein samples prior to analysis on the Agilent 2100 bioanalyzer	5989-0228EN
Glycoprotein sizing on the Agilent 2100 bioanalyzer	5989-0332EN
Using the Agilent 2100 bioanalyzer for quality control of protein samples prior to MS-analysis	5989-0771EN
Quality Control for the Agilent 2100 Bioanalyzer Protein 200 Plus LabChip Kits (technical note)	5989-3336EN

Scientific publications

The Agilent 2100 bioanalyzer is used in virtually every kind of scientific laboratory. A list of existing scientific publications with more than 2000 citations from well-known and high- impact journals is continuously updated and can be viewed at: **http://www.agilent.com/chem/2100publications**.

Agilent 2100 bioanalyzer

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Agilent Technologies

Use of the Agilent 2100 Bioanalyzer for Basmati Rice Authenticity Testing

Application

Food

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Abstract:

Ensuring integrity of raw food materials, ingredients, and products is both a product quality and regulatory compliance concern. Food suppliers and manufacturers may suffer economic and legal damages if proven to be supplying incorrectly labeled products. For example, EU Commission 1549/04 grants lower import tax on nine basmati rice varieties. A quick and cost-effective analytical method utilizing the Agilent 2100 bioanalyzer and DNA 1000 assay is shown as an alternative method to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice in ground rice products.

Introduction

The integrity of raw food materials, ingredients, and products must be maintained to ensure that they meet appropriate quality and legislative requirements. Food ingredient suppliers, manufacturers, and retailers can face legal action if proven to be supplying materials or products that are incorrectly labeled due to substitution or contamination.

One food area which has been under the spotlight in recent years is the supply of basmati rice to the UK from India and Pakistan. In Europe, Commission Regulation 1549/04 grants a lower import tax on nine basmati varieties: Basmati 370, Dehradun (Type 3), Basmati 217, Taraori, Ranbir Basmati, Kernel, Basmati 386, Pusa Basmati, and Super Basmati. Other basmati rice varieties approved by India, Pakistan, and the UK include Basmati 198, Basmati 385, Haryana Basmati, Kasturi, Mahi Suganda, and Punjab Basmati. In a Code of Practice developed by Indian, Pakistani, and UK industry and enforcement organizations that came into effect for products packed and labeled after January 2006, the level of non-basmati rice in a basmati rice product must not exceed 7% (see www.riceassociation.org.uk).

In order to check the supply of basmati rice, a DNA variety testing method using PCR amplification of eight rice microsatellite sequences has been developed for UK compliance (www.foodstandards.gov.uk/multimedia/pdfs/fsis4704basmati.pdf). During 2003, the UK Food Standards Agency carried out a surveillance exercise on basmati rice products using this method and revealed that 74% of them contained > 7% non-basmati varieties.

This study evaluates the use of the Agilent 2100 bioanalyzer to differentiate approved and non-approved varieties using three primer sets and to estimate the level of non-basmati using reference rice admixtures.

Experimental

Method

All reference basmati rice samples were obtained from the UK Food Standards Agency via the University of Wales (Bangor, UK). Other samples were from in-house basmati rice sample collections.

PCR amplification was performed using either a PE9600 or PE2400 PCR machine (Applied Biosystems).

Extraction

DNA was extracted from ground rice grains using Qiagen's DNeasy Plant Mini Kit or Promega's Maxwell 16 automated DNA extractor

PCR

DNA extracts were diluted 1 to 1 in sterile distilled water (SDW) to produce template DNA prior to use in PCRs. Amplification was performed in 25-µL PCRs containing 1x Amplitaq Gold PCR buffer (Applied Biosystems), 60 nM of each primer, 200 nM dNTPs, 3 mM $MgCl_2$, 0.05 U/µL of Ampli-Taq Gold (Applied Biosystems), and 2 µL of template DNA.

Marker	Forward Primer
RM201	CTC gTT TAT TAC CTA CAg TAC C
RM212	CCA CTT TCA gCT ACT ACC Ag
RM339	GTA ATC gAT gCT gTg ggA Ag
Marker	**Reverse Primer**
RM201	CTA CCT CCT TTC TAg ACC gAT A
RM212	CAC CCA TTT gTC TCT CAT TAT g
RM339	gAg TCA TgT gAT AgC CgA TAT g

Amplification profiles (95 °C for 15 minutes [denaturation]; 50 cycles of: 95 °C for 1 minute, 60 °C for 1 minute [amplification]; 72 °C for 10 minutes [final extension]) were used in all PCR reactions. DNA amplification was confirmed by separating PCR products using the Agilent 2100 bioanalyzer.

Capillary Gel Electrophoresis on 2100 Bioanalyzer

Reagents were prepared following manufacturers' instructions. Batches (~500 µL) of gel matrix (used to fill LabChip capillaries) were prepared as required or at 4 weekly intervals. All reagents were stored at 4 °C and allowed to reach room temperature for 1 hour before use. PCR products (1 µL)

Table 1. Analysis of Authenticated Basmati Rice Varieties Using Three Microsatellite Primer Sets

Rice variety	List of microsatellite amplification product sizes obtained with primer set*		
	RM201	RM212	RM339
Varieties listed in Commission Regulation 1549/04			
Basmati 370	162 (**162**)	134 (**139**)	200 (**193**)
Dehra Dun (Type 3)	162	134 (**139**)	200 (**195**)
Basmati 217	162	134	200
Ranbir	162	134	200
Taraori	162	134 (**139**)	200 (**195**)
Basmati 386	162	134 (**138**)	200 (**195**)
Kernel	162	134	200
Pusa	162	134 (**139**)	200 (**194**)
Super	162	134 (**140**)	204 (**196**)
Other varieties approved as basmati by UK Food Standards Agency			
Basmati 198	162	152	200
Basmati 385	162	152	200
Kasturi	162	132	166
Haryana Basmati	162	152	166
Mahi Sugandha	176	152	166
Punjab Basmati	162	152	200
Non-approved varieties			
Basmati 2000	162	152	204
Shaheen Basmati	162	152	200
Sherbati	178 (**176**)	130 (**135**)	166 (**167**)
Mugad Sugandha	178	132	166
Pak 386	178	130 (**135**)	166 (**167**)
Superfine	178	132	166
Pusa Sugandha	162	132	178
Yamini	162	134	200

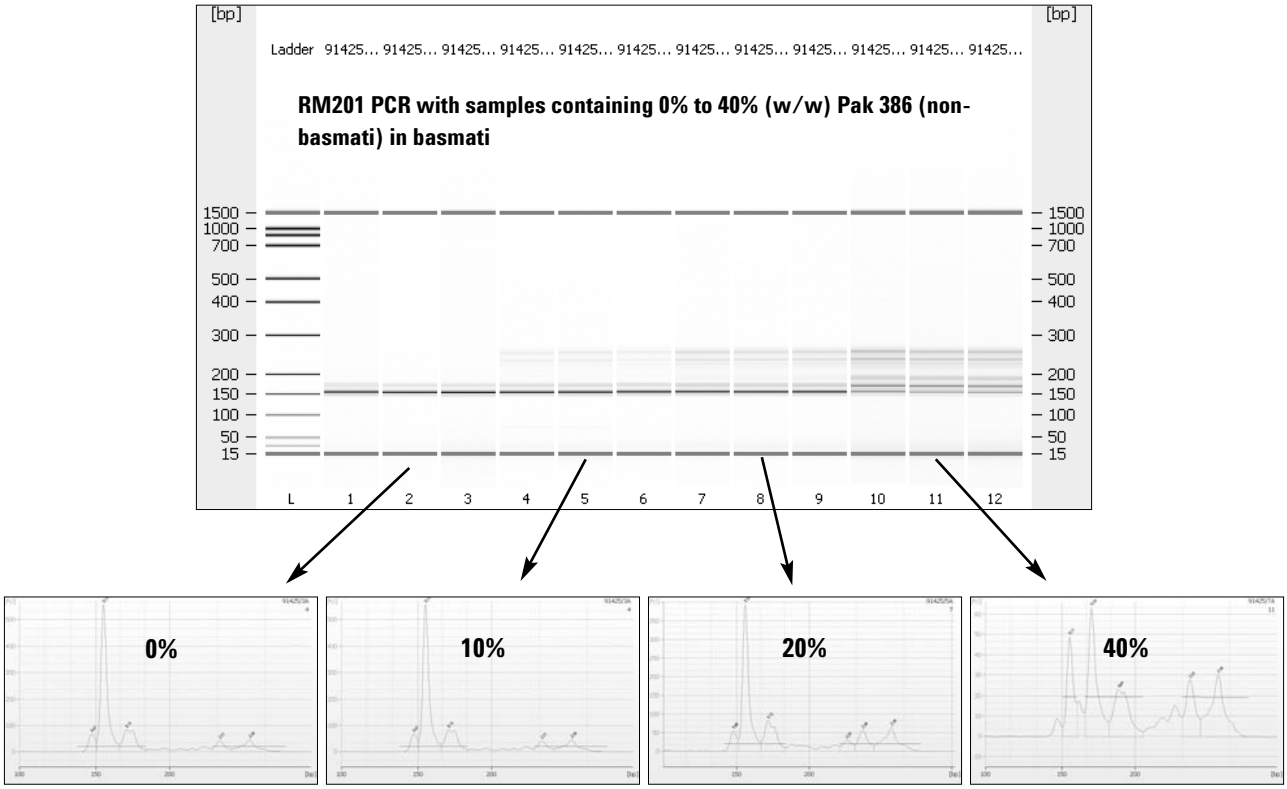
*These are from the FSA method developed by the University of Wales, Bangor. Actual size of fragments determined by the bioanalyzer using a DNA 500 chip kit are shown in brackets. The variation in bioanalyzer-determined fragment sizes can be about 5%. Shaded cells show how the varieties can be grouped using the three primer sets with analysis performed on the bioanalyzer.

were loaded directly onto prepared Series I DNA 500/DNA 1000 or Series II DNA 1000 labchips. All analysis was performed on the 2100 bioanalyzer, as per the manufacturers' instructions.

Results and Discussion

The different-sized PCR products generated when these three primer sets are applied to the different varieties are easily resolved on the bioanalyzer using the DNA 500 or DNA 1000 chip. RM 212 primers produce a 139 bp product with varieties listed in Commission Regulation 1549/04 and a 154 bp product with FSA-approved varieties apart from Kasturi and a few non-basmati varieties. The other primer sets enable separation of these varieties apart from the Yamini variety, which produces fragments similarly sized to the EC-approved varieties for all three primer sets. This variety is also difficult to distinguish from approved varieties using the standard method. The other FSA-approved basmati varieties could be distinguished from the EC-approved varieties but not from Basmati 2000 or Basmati 386 using these microsatellite primer sets. Use of further microsatellites that give PCR products that can be separated on the bioanalyzer will give improved differentiation of non-basmati rice varieties.

a) Primer set RM201



b) Primer set RM339

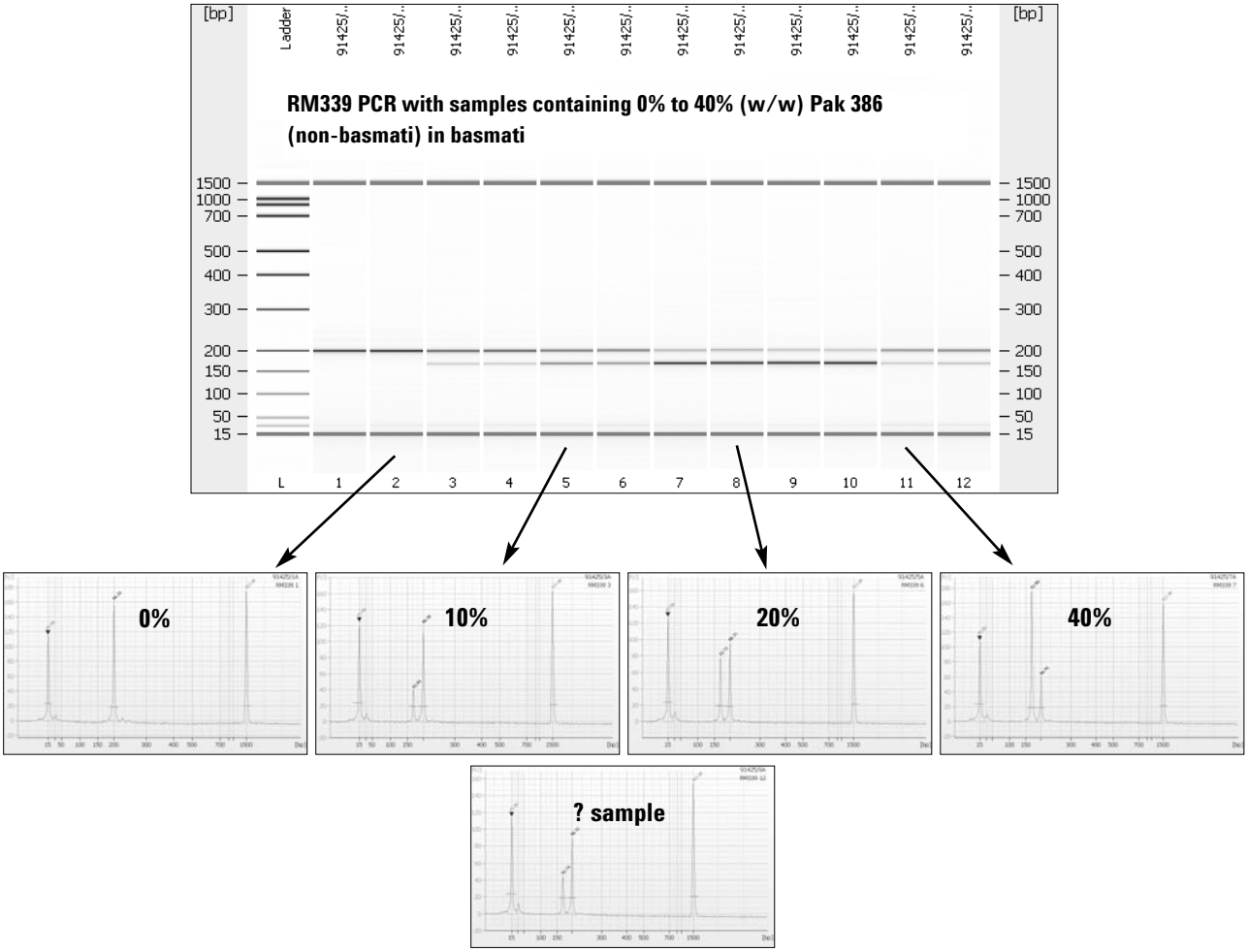


Figure 1. Analysis of non-basmati/basmati rice admixtures using two microsatellite primer sets. Estimation of percentage non-basmati variety (Pak 386) in an unknown sample.

Table 2. Experimental Summary

% Pak 386 in basmati rice	Primer RM201 mean ratio 156 bp/170 bp PCR fragments (n = 3)	Primer RM339 mean ratio 199 bp/169 bp PCR fragments (n = 2)
0	9.6	0
10	3.5	3.1
20	2.7	1.1
40	0.6	0.4
50	0.7	0.3
Unknown sample estimated to contain between 10–20% Pak 386	3.1	2.1

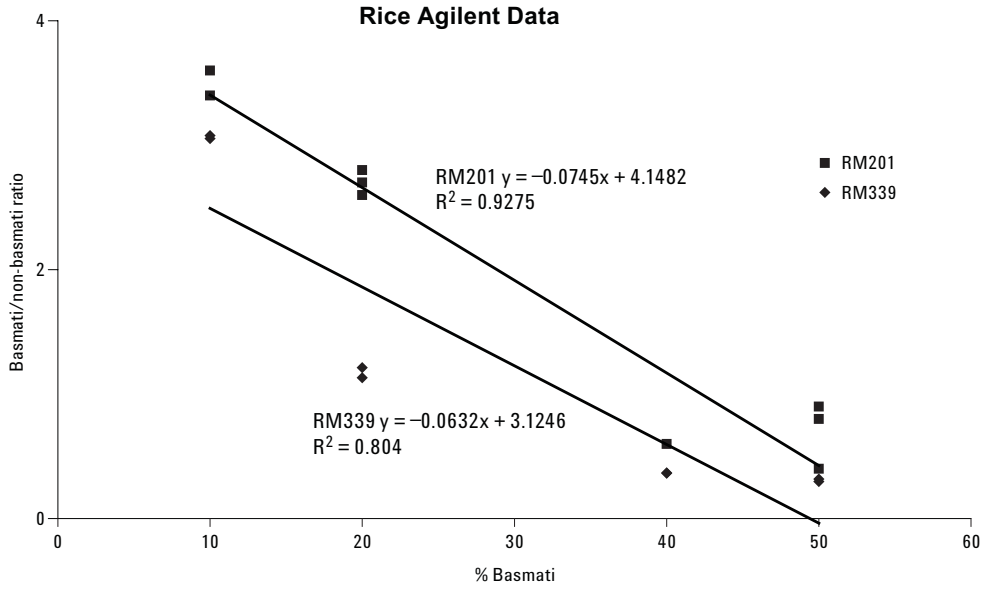


Figure 2. Standard curves based on the PCR product concentration ratios.

Both the RM201 and RM339 primer sets (Figure 1 and Table 2) could be used to produced separate and measurable PCR products with the PAK 386 basmati rice admixtures. The RM201 primers set gave a 170 bp product generated from the basmati rice and a 156 bp product from the Pak 386 variety, whereas RM339 primers gave 199 bp and 169 bp products. RM281 also gave other larger PCR products, which seemed to correlate with the presence of Pak 386 in the admixture. Standard curves (Figure 2) were produced using the PCR product concentration ratios. An unknown admixture sample was also analyzed and estimated to contain between 10 and 20% non-basmati rice in basmati.

Results show that the bioanalyzer can be used to estimate the level of non-basmati rice (Pak 386) in a basmati rice which produces different sized microsatellite PCR products from the Pak 386.

Conclusions

The Agilent 2100 bioanalyzer can be used as a quick and cost-effective alternative to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice. It should be feasible to develop further primer sets that would allow the bioanalyzer to be used to identify individual varieties. Simple manual and automated DNA extraction followed by fast PCR and post-PCR analysis on the bioanalyzer would allow rapid screening of rice materials prior to export from India and Pakistan and also allow enforcement bodies to efficiently test for microsatellite markers from non-approved basmati rice varieties in imported products.

Acknowledgements

Thanks to Mark Woolfe at UK Food Standards Agency and John Gorham and Katherine Steele at The University of Wales, Bangor, for the supply of primer set details and authentic basmati rice materials.

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Agilent Technologies

Rapid wheat varietal identification using the Agilent 2100 bioanalyzer and automated pattern-matching

Application Note

Dr Dhan Bhandari

Abstract

Accurate identification of wheat varieties is of paramount importance to the milling industry in many countries. This Application Note describes how the Agilent 2100 bioanalyzer and the Protein 230 assay can be used in conjunction with a third-party software, to analyze wheat proteins for varietal identification.

Agilent Equipment:

• 2100 Bioanalyzer Protein 230 Kit

Application Area:

• Food analysis

Agilent Technologies

Introduction

Authentication of varieties is important for the cereal industry for maintenance and testing of grain quality to meet market requirements. The charge-based separation of proteins by the acid-PAGE (polyacrylamide gel electrophoresis) technique is widely used for wheat varietal identification. However, this requires highly skilled operators to prepare, run and scan the gels and interpret band patterns. Also, there are safety concerns regarding the toxicity of unpolymerised acrylamide. While acid-PAGE is effective in analytical laboratories, the routine method can take up to two days. This is too slow for use at mill intake, which requires assessment of the wheat shipment during the period of delivery – typically under one hour. In this Application Note we demonstrate the use of the Agilent 2100 assay, with bioanalyzer and Protein 230 the Nonlinear Dynamics' Phoretix 1D Advanced (TotalLab TL120 DM) computerized pattern-recognition software to provide a better alternative to acid-PAGE. The aim of this study was to develop a robust, automated method for rapid identification of wheat varieties.

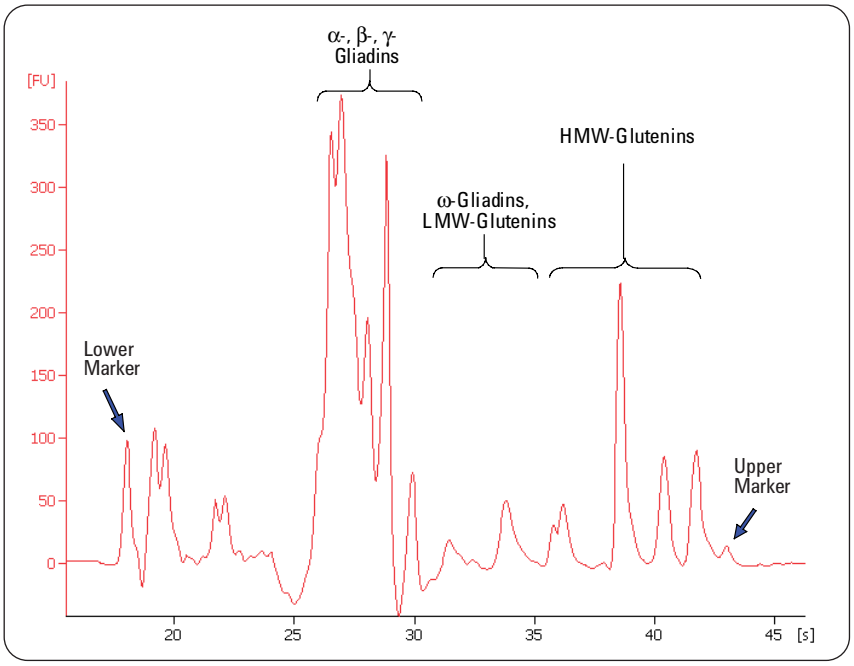

Figure 1
Typical wheat protein separation by the Protein 230 assay.

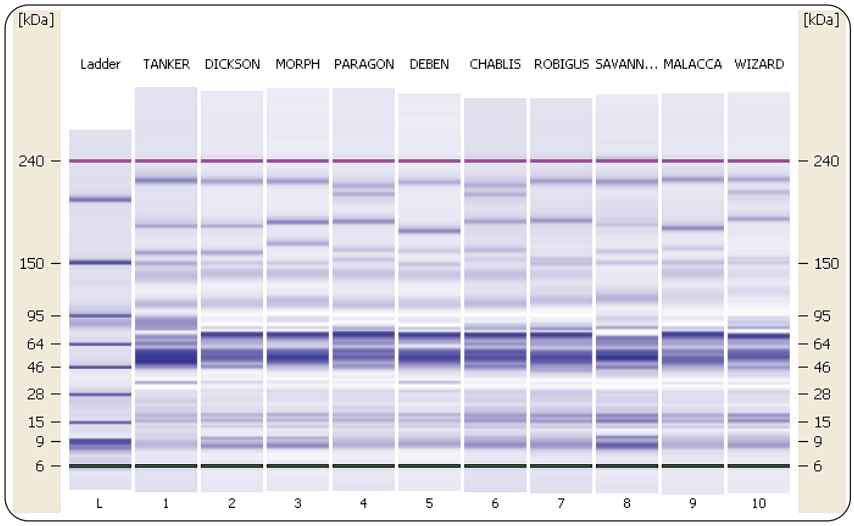

Figure 2
Bioanalyzer gel-like image of MW standards and 10 different wheat varieties.

Methods

Total wheat proteins (including glutenins) were extracted from individual grains in 0.4 mL of 2M urea, 15 % glycerol, 0.1 M DTT and 0.1 M Tris/HCl, pH 8.8, using an ultra-sonic water bath for 15 minutes. Extracts were centrifuged at 11,000 g for 5 minutes and treated with the Protein 230 assay reagents in according to the assay protocol. The samples were separated on the Agilent 2100 bioanalyzer, with each analysis of 10 samples plus ladder taking less than 25 minutes. Replicates of 34 wheat cultivars representing the 2004/5 UK Recommended List varieties were analyzed. The electropheromram profiles were processed using the Phoretix 1D Advanced and 1D Database (Nonlinear Dynamics) software for pattern-matching purposes.

Results

The Protein 230 assay produced well-resolved protein profiles, suitable for varietal discrimination (figures 1 & 2). The Phoretix software was able to compare the electropherogram profiles. Figure 3 shows an example of a dendrogram where all the replicates of three different varieties are correctly grouped. A prototype wheat library was developed by selecting the most representative varietal profile. Results showed that 90 % of test samples could be identified within the top three matches. Work is in progress to optimize the performance of this library. The practicality of the method and the robustness of the system is bourne out by the fact that the system is now in routine use in UK commercial mill intake laboratories.

Conclusions

Our study has demonstrated that using the Agilent 2100 bioanalyzer with the Phoretix system offers a standardized, objective method for rapid varietal discrimination. The ease of use and total analysis time of less than 50 minutes makes it most suitable for mill intake use. The optimized system will enable millers to make more confident decisions in accepting grain consignments, and could become widely adopted as an effective policing tool within the grain industry. A number of UK mills have purchased the combined systems for screening wheat deliveries at intake.

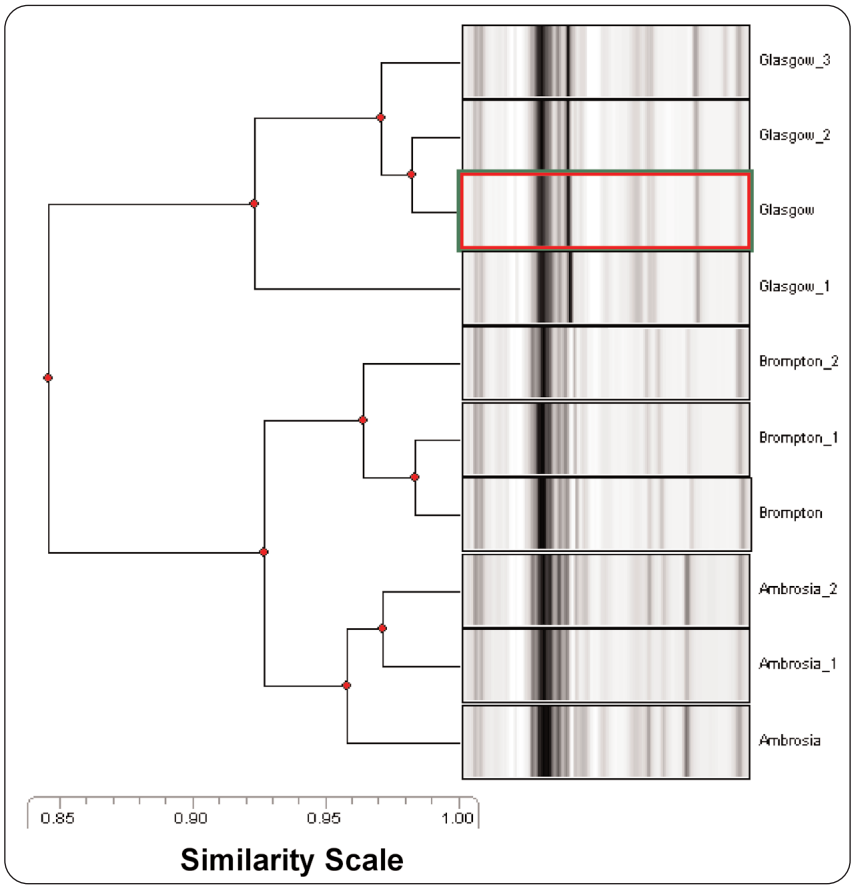

Figure 3
Dendrogram illustrating pattern-matching of replicate profiles of 3 wheat varieties.

Acknowledgements

CCFRA is grateful to the National Association of British and Irish Millers (nabim) for sponsoring this study.

Dr Dhan Bhandari is Senior Scientist at Campden & Chorleywood Food Research Association (CCFRA), Chipping Campden, Gloucestershire, GL55 6LD, UK.

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Agilent Technologies

Strawberry and Raspberry Fruit Differentiation Using the Agilent CE 2100 Bioanalyzer

Application Note

Food

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Abstract

Food authenticity is an important and rapidly expanding area that requires development of molecular approaches to help avert fraudulent replacement of expensive food ingredients, and to ensure correct ingredient levels are present in prepacked foods. We report here on the development of a method that uses polymerase chain reaction (PCR) and the DNA 1000 chip to distinguish between DNA derived from the fruits of strawberry and raspberry. Using this approach, characteristic profiles from strawberry and raspberry DNA are generated on the Agilent 2100 capillary electrophoresis system, which may prove useful in food authentication studies.

Agilent Technologies

Introduction

Food authentication is an important and rapidly expanding analytical area that is needed to ensure that food conforms to current international legislation and that policies on food labeling and ingredients are enforced.

Raspberry (genus: *Rubus*) is used in many foods, including purées, jellies, jams, pies, cakes, pastries, dessert toppings, juices, wines, and dairy products such as ice cream and yogurt, as well as being eaten fresh or stored frozen for consumption later. The leaves of raspberry are often used in herbal teas, and the fruit is also used for potential health benefits. Strawberry (genus: *Fragaria*) is also a common type of fruit that is cultivated worldwide and is of global economic significance.

However, recent studies [1, 2] report that some food and drinks (fruit juices in particular) labeled as containing a particular fruit, contain little or no fruit of that particular species, or may have substituted or mixed that fruit with other edible fruits. This may occur through either deliberate adulteration or unintentional processing errors (via contamination through inefficient washing procedures or coprocessing of fruits). Such instances are in contravention of the law, and stakeholders (food retailers, enforcement agencies, etc.) all require access to methods that allow the accurate identification of food ingredients to ensure regulatory compliance and protect consumers. Additionally, correct identification of ingredients in food products is needed to support the authentic composition of food, especially in relation to the declared presence of allergens in a food product, and also in the fraudulent replacement of more expensive food ingredients.

A novel application of the Agilent 2100 capillary electrophoresis (CE) system to differentiate between DNA derived from strawberry and raspberry fruits, is reported here. This approach makes use of microsatellite markers that allow differentiation of strawberry and raspberry DNA based on presence/absence or size differentiation of PCR products, easily measured using the DNA 1000 chip.

Experimental

PCR Primers

Microsatellite markers that have been previously described [3] were used to differentiate between strawberry and raspberry DNA:

Fvi11 Forward: GCATCATCGTCATAATGAGTGC
Fvi11 Reverse: GGCTTCATCTCTGCAATTCAA

Fvi20 Forward: GAGTTTGTCATCCTCAGACACC
Fvi20 Reverse: AGTGACCCAGAACCCAGAA

Samples

Authenticated DNA for Samples A and B were kindly provided by the SCRI (Dundee, UK) and consisted of strawberry samples derived from a numbered selection from a commercial breeding program, and Glen Moy (raspberry), respectively.

Raspberries (Sample D) were bought from a UK supermarket chain as prepacked fruit (225 g) labeled as raspberries (produce of Spain). Strawberries (Sample C) were purchased from the same UK supermarket store at the same time, as prepacked fruit labeled as strawberries. Two individual DNA extractions were taken from each fruit batch and labeled as Samples C1 and C2, and Samples D1 and D2.

DNA Extraction

For Samples C and D, DNA was extracted from strawberry and raspberry fruits using a cetyl trimethylammonium bromide (CTAB) buffer (50 mM tris HCl; 4 M NaCl, 1.8% CTAB; 25 mM EDTA). Approximately 100 mg fruit samples were weighed and homogenized, DNA was extracted using the above CTAB buffer, resuspended in 100 µL of 1x TE buffer, and quantified using a spectrophotometer.

Thermal Cycle Conditions

25 µL PCR reaction mixes were made based on the following components: 12.5 µL of 2x Fast Start PCR Master mix (Product number 04710436001, Roche); 300 nM of appropriate forward primer; 300 nM of appropriate reverse primer; 15 ng of extracted genomic DNA; and sterile distilled water to make a final volume of 25 µL.

Thermal cycle conditions (MJ Research Tetrad #2 PCR machine) consisted of 95 °C for 6 min; 40 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 10 min and hold at 4 °C.

Use of the DNA 1000 Chips on the Agilent 2100 CE System

All chips were prepared according to the instructions provided with the Agilent DNA 1000 LabChip kit. The gel-dye mix was prepared by mixing 400 µL of the gel matrix with 20 µL of the dye concentrate, then filtering the mixture through a spin filter. The separation chip was filled with the gel matrix/dye mixture, and 5 µL of the markers was added to each sample well. After adding samples (1 µL each) to the sample wells and the DNA sizing ladder (1 µL) to the assigned ladder well, the chip was vortexed and run on the Agilent 2100 bioanalyzer.

Results and Discussion

Fvi11 Assay

According to published literature [3, 4], Fvi11 is based on a $(GA)_{16}$ repeat motif and should give an amplicon of around 137 bp in length with strawberry, but also exhibits polymorphism in amplicon size between *Fragaria* varieties. However, Fvi11 should not cross-react with raspberry, and so no amplicon should be present.

The results from this preliminary study shown in Figures 1A and 1B indicate that Fvi11 gives an amplicon of around 122 bp in the samples that contained strawberry (A and C). Additional amplicons were also sometimes observed at 282 and 290 bp, but within the confines of the limited experimental data presented here, not on a repeatable basis. In line with expectations, Figures 1A and 1C show that Fvi11 did not cross-react with samples that contained raspberry (B and D).

Negative controls showed no detectable amplification. Additionally, Fvi11 showed a positive result and amplified the same 122-bp fragment when tested on a commercially available strawberry sauce sample with a listed ingredient of 40 percent whole strawberries (Figure 1D), inferring the assay's applicability to processed food samples containing fruit.

Fvi20 Assay

According to published literature [3, 4], microsatellite marker

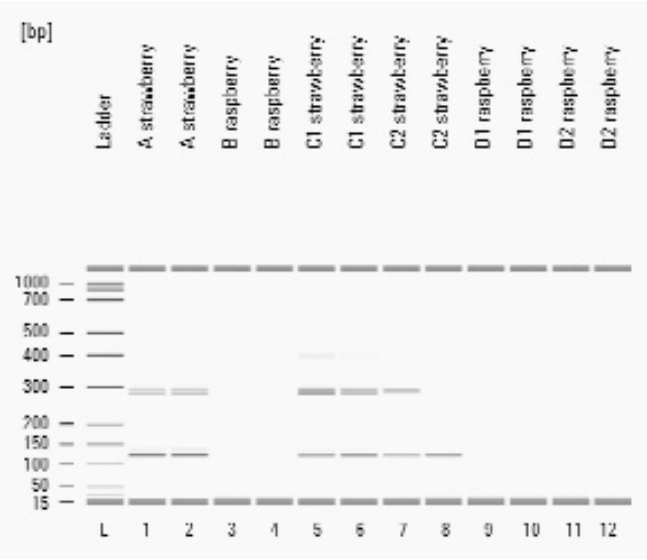


Figure 1A: Gel-like image based on Fvi11 assay on all samples.

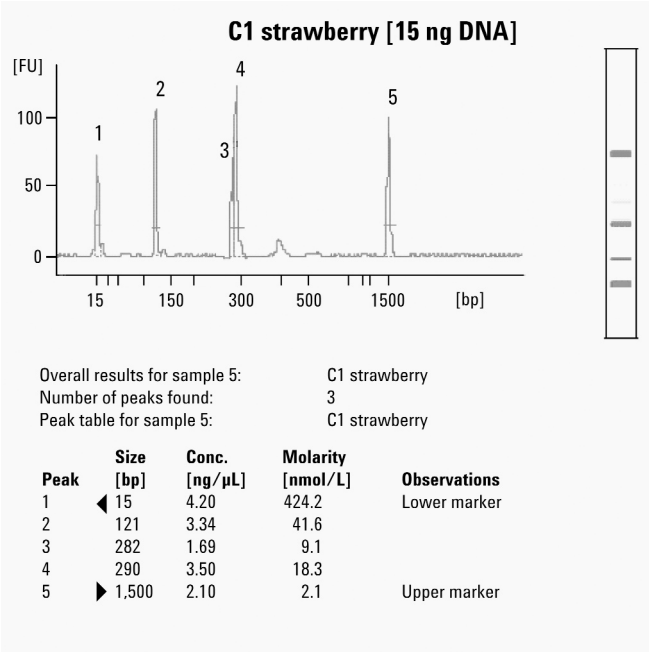


Figure 1B. Electropherogram to show profile generated using Fvi11 with strawberry DNA.

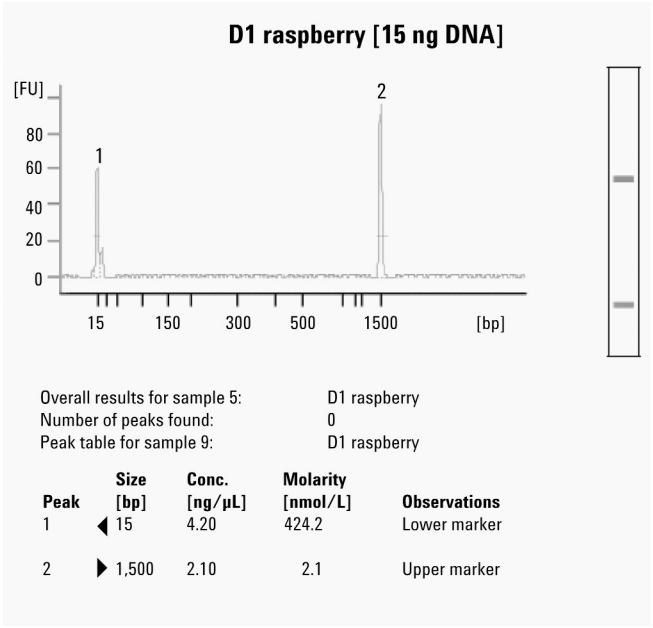
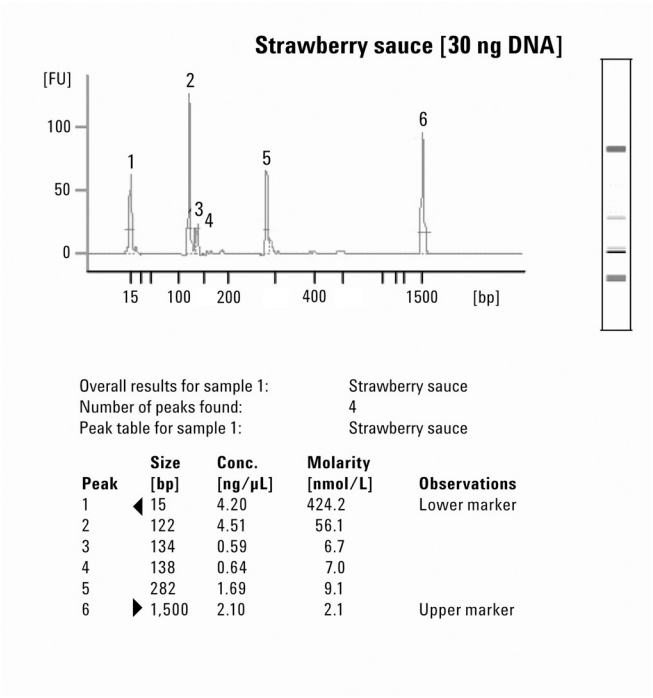


Figure 1C. Electropherogram to show absence of bands when using Fvi11 with raspberry DNA.



Overall results for sample 1: Strawberry sauce
Number of peaks found: 4
Peak table for sample 1: Strawberry sauce

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	122	4.51	56.1	
3	134	0.59	6.7	
4	138	0.64	7.0	
5	282	1.69	9.1	
6	▶ 1,500	2.10	2.1	Upper marker

Figure 1D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi11 to a strawberry sauce sample.

Fvi20 is based on a $(GA)_{20}$ simple sequence repeat motif sequence, and has been shown to give a single or multiple bands around 162 bp in length for *Fragaria* varieties, but only a single amplicon with raspberry (*Rubus*) varieties.

Based on the results from this preliminary study, shown in Figures 2A and 2B, the application of the Fvi20 assay to samples that contained strawberry (A and C) gave single or multiple amplicons at around 144, 162, and/or 175 bp. In the limited strawberry samples tested in this study, the 144-bp fragment was present and predominated, while the occurrence of the other bands was less repeatable.

The application of the Fvi20 marker locus to samples that contained raspberry (B and D), shown in Figures 2A and 2C, showed the presence of a single band at 136 bp, which was easily distinguished from the 144-bp amplicon characteristic of strawberry cultivars.

Negative controls and extraction blanks showed no detectable amplification. Furthermore, the application of Fvi20 to the commercially available strawberry sauce sample showed bands around 143 and 161 bp, characteristic of strawberry DNA being present (Figure 2D).

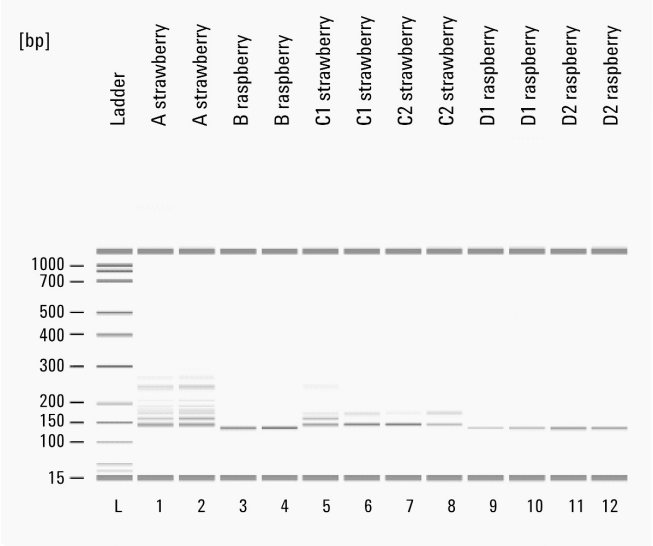
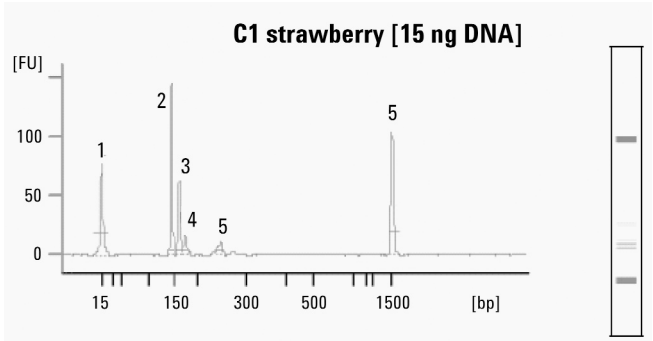


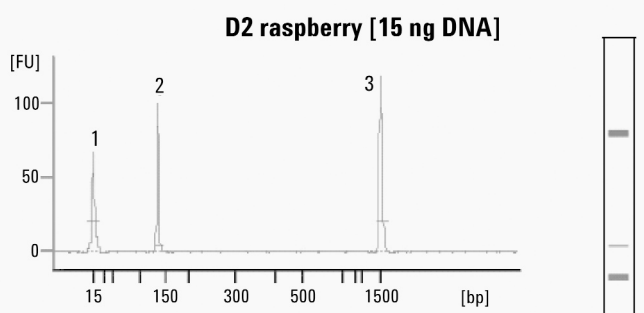
Figure 2A. Gel-like image based on Fvi20 assay on all samples.



Overall results for sample 5: C1 strawberry
Number of peaks found: 3
Peak table for sample 5: C1 strawberry

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	144	4.13	43.6	
3	161	2.01	18.9	
4	174	0.76	6.6	
5	248	0.60	3.7	
6	▶ 1,500	2.10	2.1	Upper marker

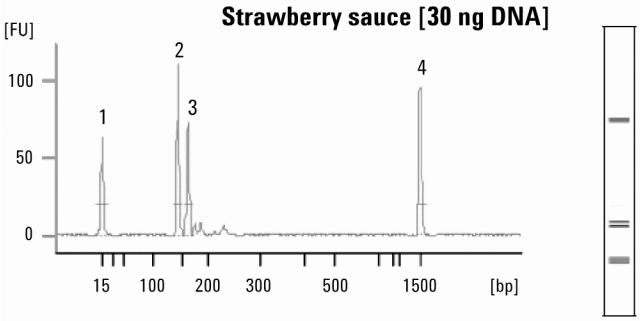
Figure 2B. Electropherogram to show profile generated using Fvi20 with strawberry DNA.



Overall results for sample 5: D2 raspberry
Number of peaks found: 1
Peak table for sample 12: D2 raspberry

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	135	2.44	27.4	
3	▶ 1,500	2.10	2.1	Upper marker

Figure 2C. Electropherogram to show presence of one band when using Fvi20 with raspberry DNA.



Overall results for sample 1: Strawberry sauce
Number of peaks found: 2
Peak table for sample 1: Strawberry sauce

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	143	3.71	39.4	
3	161	3.01	28.2	
4	▶ 1,500	2.10	2.1	Upper marker

Figure 2D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi20 to the strawberry sauce sample.

Conclusions

Originally, the microsatellite markers of Fvi11 and Fvi20 were used to assess genetic variability in strawberry (*Fragaria*) varieties. We have shown the novel application of these primer pairs using PCR and the Agilent CE 2100 system, to differentiate between samples that contain strawberry and raspberry DNA. Based on initial studies and amplicon sizes, these may prove useful in food authentication studies. The results from this small study are only representative of specific varieties of *Fragaria* and *Rubus*, but demonstrated clear differentiation between strawberry and raspberry DNA using traditional PCR followed by resolving the PCR products on the Agilent 2100 CE system.

References

1. Food Commission Survey results, The Food Magazine, 25 February 2008, http://www.foodmagazine.org.uk/press/food_flavourings/
2. A. Stój and Z. Targoński, "Use of amino acid analysis for estimation of berry juice authenticity," *Acta Sci. Pol., Technol. Aliment* (2006) 5(1):61–72
3. M.V. Ashley, J.A. Wilk, S.M.N. Styan, K.J. Craft, K.L. Jones, K.A. Feldheim, K.S. Lewers, T.L. Ashman, "High variability and disomic segregation of microsatellites in the octoploid *Fragaria virginiana* Mill. (Rosaceae)," *Theor. Appl. Genet.* (2003) 107:1201–1207
4. K.S. Lewers, S.M.N. Styan, S.C. Hokanson, "Strawberry GenBank-derived and Genomic Simple Sequence Repeat (SSR) Markers and Their Utility with Strawberry, Blackberry, and Red and Black Raspberry," *J. Amer. Soc. Hort. Sci*. (2005) 130(1):102–115

Acknowledgements

We gratefully acknowledge provision of strawberry Sample A and raspberry Sample B as authenticated samples from Julie Graham, SCRI, for the purposes of this study.

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Agilent Technologies

Analysis of the Purity and Storage Stability of L-Ascorbic Acid Powders

Application Note

Author

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Introduction

Most previous studies of the storage stability of L-ascorbic acid were done on solutions of the acid. However, L-ascorbic acid is usually kept in the dry powder form, batches of which may contain breakdown products or other impurities arising from the production process. The presence of these contaminants is suspected to adversely affect the stability of the acid and/or contribute to color changes in the stored product over time.

This note describes a trial of the purity and stability of stored L-ascorbic acid powder samples from four different manufacturers, and assesses degradation compounds and/or precursors from the production process. Ion suppression reversed phase HPLC was used, with PLRP-S columns. PLRP-S is a rigid macroporous styrene/divinylbenzene HPLC phase with outstanding chemical and physical stability with acidic eluents.

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Materials and Reagents

Samples: L-ascorbic acid powders from different manufacturers produced from 1971 to 1987
Impurity reference compounds: L-dehydroascorbic acid (DHAA), 2,3-diketo-L-gulonic acid, L-threonic acid, oxalic acid, furfural, 5,6-*O*-isopropylidene-L-ascorbic acid, D-gulonic acid-1,4-lactone

Conditions

Column: PLRP-S 100Å 5 µm, 150 x 4.6 mm (p/n PL1111-3500)
Eluent: 0.2 M NaH_2PO_4, pH 2.14
Flow rate: 0.5 mL/min
Detector: UV, 268 and 220 nm

Results and Discussion

A plot of manufacturing date against purity is given in Figure 1, showing a gradual decline in L-ascorbic acid with age even in dry powder formulations. Comparison of the retention times of sample peaks with those of the reference compounds (Table 1) did not reveal any sign of degradation products or precursors associated with the manufacture or storage of L-ascorbic acid. The absence of these compounds suggests that other degradation routes are present when L-ascorbic acid is stored under near anaerobic conditions of low water activity.

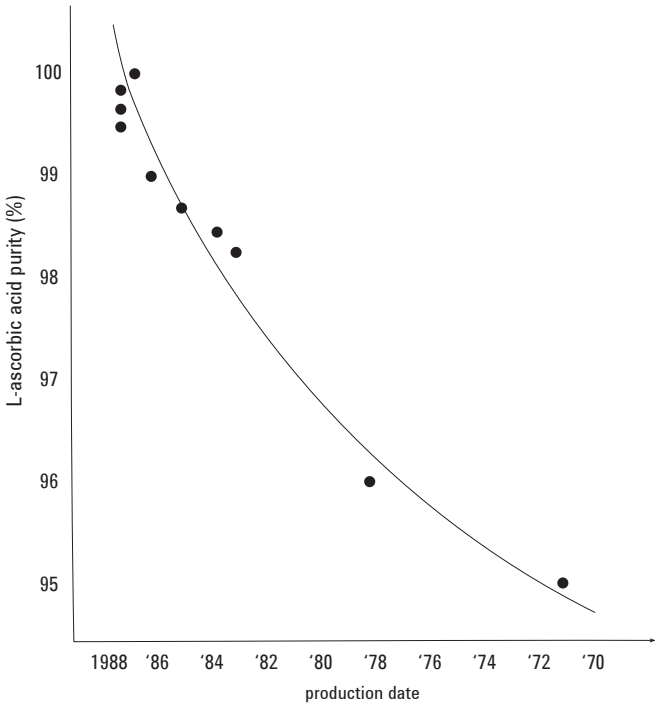

Figure 1. Graph of L-ascorbic acid purity and production date of powder samples of L-ascorbic acid.

Table 1. Retention times of reference compounds.

Sample	L-ascorbic acid association	Retention time (min)	Limit of detection (µmol)
oxalic acid	degradation product	6.00	0.039
L-threonine	degradation product	6.30	0.053
2,3-diketo-L-gulonic acid	precursor	6.60	-
D-gulonic acid-1,4-lactone	precursor	6.60	-
L-dehydroascorbic acid	degradation product	7.40	0.017
L-ascorbic acid	-	8.70	
5,6-*O*-isopropylidene-L-ascorbic acid	precursor	8.70	
fufural	degradation product	12.10	

The samples varied in their degree of whiteness regardless of the date of production, suggesting that differences in whiteness were not indicators of purity but may have been be due to differing production methods. However, the aged samples manufactured in 1971 and 1978 had a brown appearance, most evident in the 1978 sample. Measurement of the L-ascorbic acid content of these samples was lower compared to newer samples, but no impurities were detected.

The oxidative degradation of a solution of L-ascorbic acid was assessed, following the pathway shown in Figure 2.

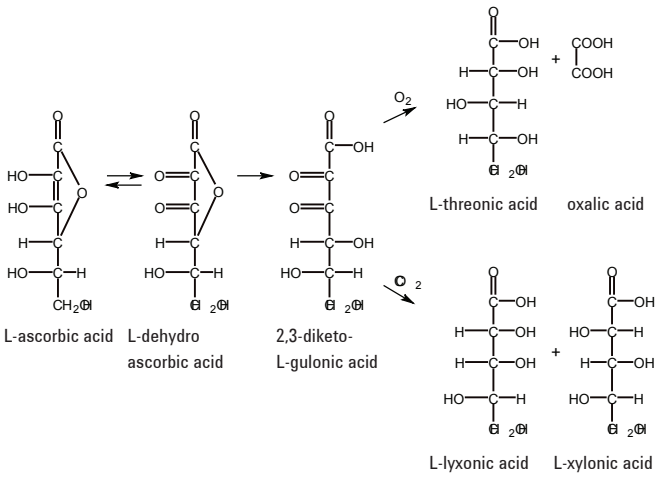

Figure 2. The proposed oxidative degradation path of L-ascorbic acid.

Table 1 shows the minimum limits of detection of the degradation products L-dehydroascorbic acid, oxalic acid and L-threonic acid from this assay. It was not possible to carry out a test for 2,3-diketo-L-gulonic acid because a pure reference compound was not available.

The complete data set and analysis is available in Kennedy *et al.* (1989).

Conclusion

Ion suppression HPLC using PLRP-S columns successfully elucidated the effects of age on the gradual deterioration of dry powder L-ascorbic acid, and suggested the presence of alternative degradation mechanisms when the acid is stored in dry powder form.

Reference

Kennedy, JF, Rivera, ZS, Lloyd, LL, Warner, FP, White, CA (1989) The use of reversed-phase HPLC for the determination of purity and stability on storage of L-ascorbic acid samples. *J. Micronutr. Anal.,* 5, 281-289.

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Agilent Technologies

Identification of different meat species by the Agilent Fish ID solution on the Agilent 2100 Bioanalyzer

Application Note

Food Authenticity, Food Testing

Authors

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Abstract

In food processing plants, a large number of various ingredients originating from different animal species are used as part of the production process. Sometimes this is done on the same production line. The Agilent Fish ID solution was developed to identify the species of fishes from food samples of different processing levels. This application note investigates its abilities to detect the presence of non-fish species, specifically mammalian or avian DNA originating from dairy products or meat.

Introduction

The Agilent Fish ID solution was introduced to allow fast and easy identification of fish species from raw, cooked, or otherwise processed fish samples. Food manufacturers are using a large number of ingredients in the preparation of their products. This includes dairy products and meat from various mammalian or avian species. These can be part of seafood preparations, either as wanted ingredient or as a contamination from the production process. The aim of this study was to determine the ability of the Fish ID solution to detect mammalian or avian DNA using meat samples.

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Materials and Methods

Meat samples

Beef, pork, wild boar, lamb, chicken, turkey, and duck samples were obtained from local supermarkets or butchers.

Isolation of DNA from meat tissue

DNA was isolated using the protocol described in the Agilent DNA Isolation kit which comes as part of the Fish ID Ensemble. DNA concentrations and 260/280, 260/230 ratios were checked using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

Amplification of *Cyt*b target sequence

For the amplification of the *Cyt*b target sequence, 1 µl of the isolated DNA was used according to the protocol of the Agilent PCR-RFLP Fish ID kit. As a positive control, the kit supplied *Salmo salar* DNA was also amplified as described in the manual. Successful amplification of all samples as well as a clear negative control (NTC) was validated using a DNA 1000 assay on the Agilent 2100 Bioanalyzer.

Restriction digestion of the PCR products

Following the protocol of the Agilent PCR-RFLP Fish ID kit, a 2.5 µL aliquot of the PCR reaction was used in the restriction digestion with each of the kit supplied enzymes *Dde* I, *Hae* III, and *Nla* III.

Analysis of restriction patterns using the Bioanalyzer

The digested samples and the positive control salmon DNA were run on a DNA 1000 chip according to protocol. For each sample, the three independent digests were loaded in consecutive wells, allowing the analysis of four samples per chip. The resulting electropherograms were analyzed using the 2100 Bioanalyzer Expert software.

Results

DNA of sufficient yield and quality was prepared from all tissue samples using the Agilent DNA Isolation kit provided as part of the Fish ID solution. Two independent DNA samples for each of the meat tissues were used in the PCR together with the salmon positive control DNA supplied by the kit and a no template control. The results of the PCR reactions were validated on the 2100 Bioanalyzer using a DNA 1000 assay (Figure 1). For mammalian samples, a single clear PCR product was obtained similar to the salmon positive control. Turkey showed a reproducible double band, whereas chicken and duck samples resulted in a large number of unspecific amplicons present in the reaction.

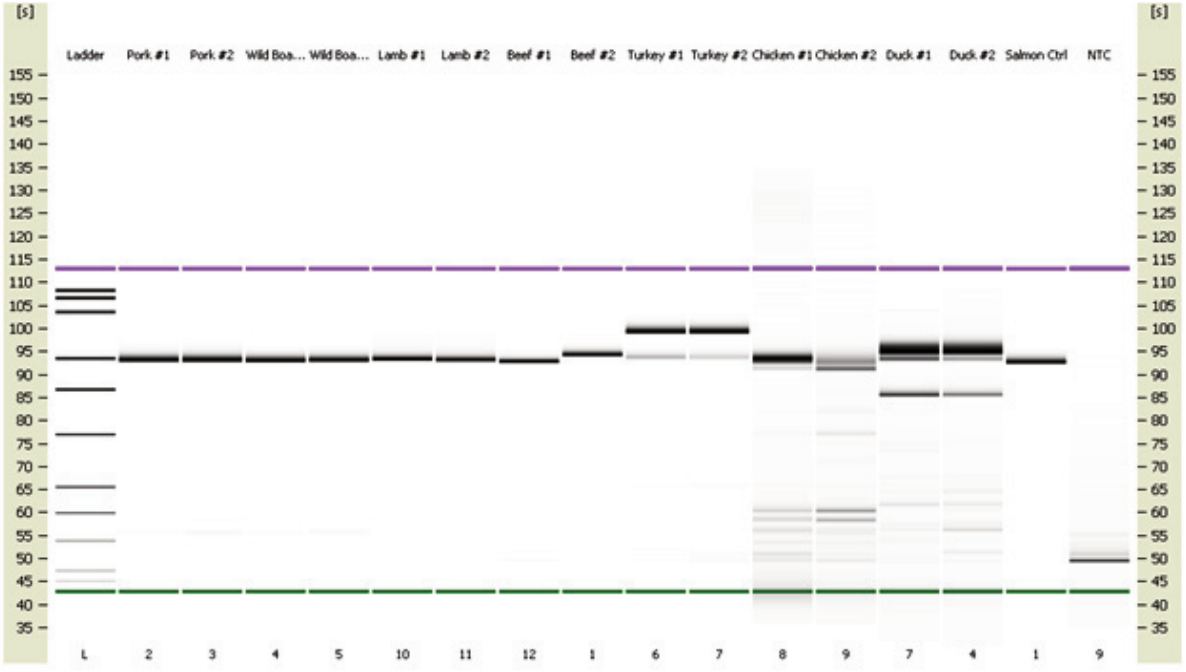

Figure 1: ***Analysis of Cytb PCR results.*** *Two independent meat DNA samples, the salmon (S. salar) positive control and a no-template control were analyzed on a Bioanalyzer using a DNA1000 assay. The virtual gel image shows a comparison file assembled of all samples using the Expert software. Lane 1: Ladder, lane 2 + 3: Pork, lane 4 + 5: Wild boar, lane 6 + 7: Lamb, lane 8 + 9: Beef, lane 10 + 11: Turkey, lane 12 + 13: Chicken, lane 14 + 15: Duck, lane 16: S. salar positive control, lane 17: NTC.*

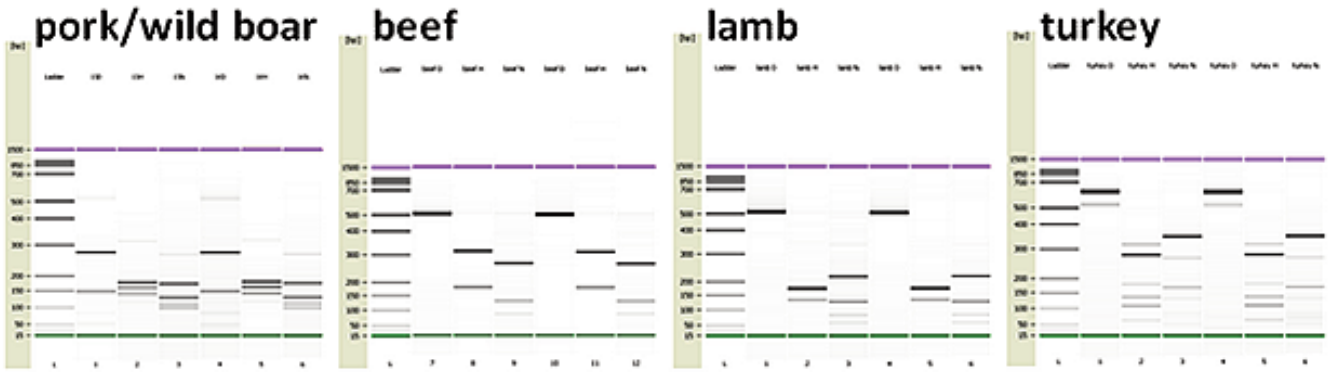

Figure 2. Restriction digestion analysis on the Agilent 2100 Bioanalyzer. The virtual gel images show the pattern combination for the restriction digestion of PCR products obtained from two independent meat tissue samples of pork, beef, lamb and turkey. Each panel shows a lane containing the ladder, Dde I, Hae III, and Nla III digestion pattern for sample 1, followed by sample 2 for the same tissue. Pork and wild boar resulted in the same pattern, therefore only the pork results are shown.

All PCR reactions, with the exception of the NTC, were subsequently used in a restriction digestion according to the manual of the PCR-RFLP Fish ID kit. As expected from the PCR results, both chicken and duck resulted in a multitude of fragments not producing a conclusive pattern (data not shown). In mammalian tissue and turkey, unique and easily identifiable patterns could be obtained (Fig. 2). Pork and wild boar gave rise to the same pattern combination. Fragment sizes are summarized in Table 1.

Table 1. Fragment Sizes and Standard Deviation in Bp for Pork, Wild Boar, Beef, Lamb and Turkey. The 132 Bp Fragment in Brackets was Consistently Present in Nla III Digests of Turkey but was not Detected with Default Settings in the Expert Software as it was Below the Peak Threshold

	***Dde* I**	***Hae* III**	***Nla* III**
Pork	276 ± 0.6, 149 ± 0.6	180 ± 1.3, 162 ± 1.3, 142 ± 1.7	175 ± 1.0, 130 ± 0.5, 109 ± 1.3, 98 ± 0.8
Wild boar	274 ± 1.0, 149 ± 0.8	181 ± 1.5, 162 ± 1.0, 143 ± 2.0	174 ± 0.8, 130 ± 0.8, 111 ± 1.0, 99 ± 0.6
Beef	505 ± 6.0	313 ± 3.1, 180 ± 0.6	267 ± 1.7, 131 ± 0.6, 87 ± 0.5
Lamb	517 ± 4.2	178 ± 1.0, 171 ± 1.0, 135 ± 1.0	220 ± 1.3, 129 ± 1.0, 84 ± 0.8, 58 ± 0.5
Turkey	624 ± 0.7, 525 ± 0.7	319 ± 0.0, 280 ± 1.4, 180 ± 1.4, 137 ± 0.7, 110 ± 0.7, 64 ± 0.7	352 ± 1.4, 271 ± 0.7, 169 ± 0.7, (132 ± 0.7)

Summary

Due to the design of the primers to cover a wide range of fish species, it was expected that non-fish species would be detected. A small scale validation with a few common mammalian and avian meat tissues showed the capability of the kit to successfully identify pork, beef and lamb. Avian *Cyt*b target DNA on the other hand seems to differ substantially from its mammalian or fish homolog. Only turkey gave rise to a simple, easily identifiable pattern, although producing two different PCR products. A full scale meat species identification solution will require a different primer design or even a different target sequence.

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Agilent Technologies

Agilent DNA Fish ID solution

Discrimination of Different Meat Species by the Agilent Fish ID Solution on the Agilent 2100 Bioanalyzer System

FOOD AUTHENTICITY

A quick, robust and easy to use protocol to identify the species based on a well accepted PCR-RFLP method allows analysis from sample (includes most processed meats and fish) to result in one working day and yields good discrimination results

In food processing plants, a large number of various ingredients originating from different animal species are used as part of the production process. Sometimes this is done on the same production line. The Agilent Fish ID solution was developed to identify the species of fishes from food samples of different processing levels. Here, we look at its abilities to detect the presence of non-fish species, specifically mammalian or avian DNA originating from dairy products or meat.

DNA based testing methods allow sensitive detection and identification from almost all but the most heavily processed food samples.

The Agilent DNA Fish ID ensemble provides all reagents and enzymes to complete a PCR-RFLP method for a Cytb PCR target sequence and analysis of restriction fragment patterns on the Agilent 2100 Bioanalyzer to identify fish species based on this mitochondrial sequence. The availability of commercial screening solutions allows for more reliable and robust test results through well-matched components and facilitates testing for screening purposes by the use of mastermix formulations and streamlined protocols. The Agilent DNA Fish ID solution was evaluated for the purpose of identifying the species of other meat products that may be intentionally or inadvertently mixed into a finished product. Good results were obtained for beef, pork, wild boar, lamb and turkey.

Table 1: Fragment sizes and standard deviation in bp for pork, wild boar, beef, lamb and turkey. The 132 bp fragment in brackets was consistently present in Nla III digests of turkey but was not detected with default settings in the Expert software as it was below the peak threshold.

	***Dde* I**	***Hae* III**	***Nla* III**
Pork	276 ± 0.6, 149 ± 0.6	180 ± 1.3, 162 ± 1.3, 142 ± 1.7	175 ± 1.0, 130 ± 0.5, 109 ± 1.3, 98 ± 0.8
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Turkey	624 ± 0.7, 525 ± 0.7	319 ± 0.0, 280 ± 1.4, 180 ± 1.4 , 137 ± 0.7, 110 ± 0.7, 64 ± 0.7	352 ± 1.4, 271 ± 0.7, 169 ± 0.7, (132 ± 0.7)

Key Benefits

- Agilent 2100 Bioanalyzer has the widest range of assays available on the market covering applications for RNA, DNA or protein
- Fast, reproducible and robust method replacing gel electrophoresis
- Agilent method using convenient mastermix format reagents will have you identifying your fish within hours, no fish standard or guesswork required.
- Flexible modular system: In-house accredited methods can replace individual building blocks of the Fish ID solution
- Overlay to the Expert software allows rapid identification of restriction fragments and close matches.
- Agilent method is now useful for testing some other meats such as beef, pork, lamb, wild boar or turkey as well as most fresh or processed fish.

Discrimination of Different Meat Species by the Agilent Fish ID Solution on the Agilent 2100 Bioanalyzer System

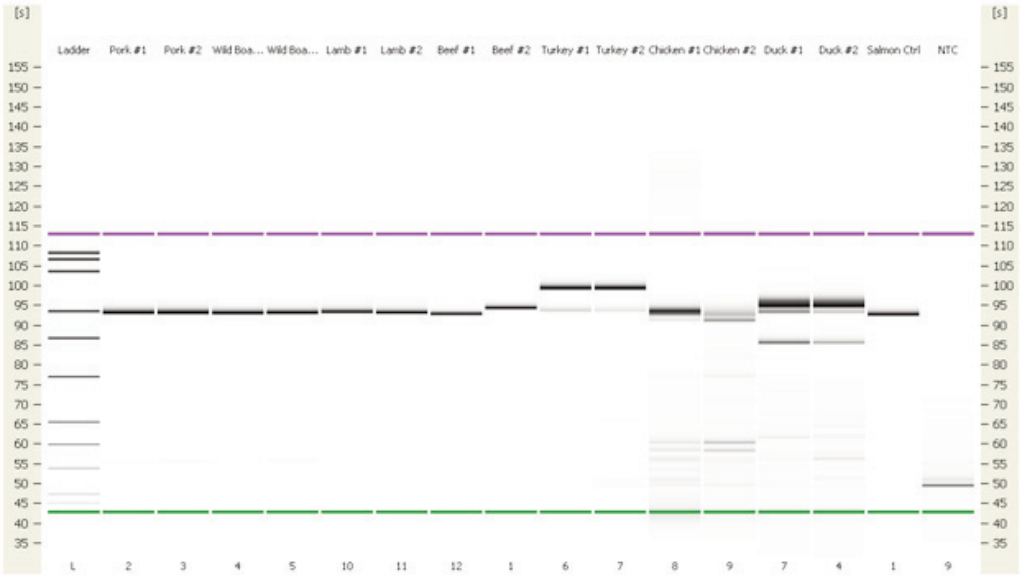

Figure 1: Analysis of Cytb PCR results. Two independent meat DNA samples, the salmon (S. salar) positive control and a no template control were analyzed on a Bioanalyzer using a DNA1000 assay. The virtual gel image shows a comparison file assembled of all samples using the Expert software. Lane 1: Ladder, lane 2 + 3: Pork, lane 4 + 5: Wild boar, lane 6 + 7: Lamb, lane 8 + 9: Beef, lane 10 + 11: Turkey, lane 12 + 13: Chicken, lane 14 + 15: Duck, lane 16: S. salar positive control, lane 17: NTC.

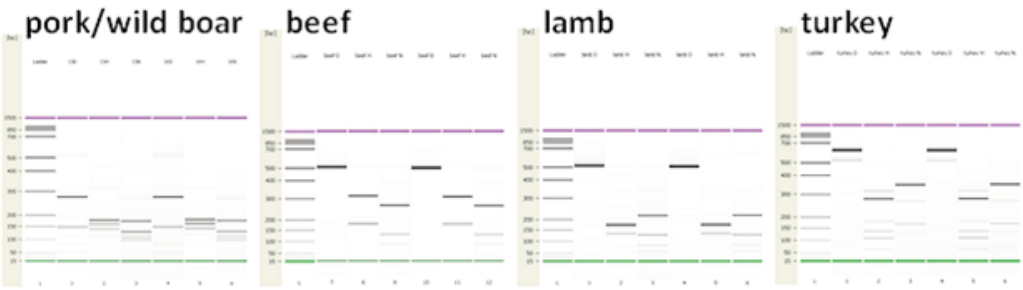

Figure 2. Restriction digestion analysis on the Agilent 2100 Bioanalyzer. The virtual gel images show the pattern combination for the restriction digestion of PCR products obtained from two independent meat tissue samples of pork, beef, lamb and turkey. Each panel shows a lane containing the ladder, Dde I, Hae III, and Nla III digestion pattern for sample 1, followed by sample 2 for the same tissue. Pork and wild boar resulted in the same pattern, therefore only the pork results are shown.

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Agilent Technologies

Determination of Melamine Residue in Milk Powder and Egg Using Agilent SampliQ Polymer SCX Solid Phase Extraction and the Agilent 1200 Series HPLC/UV

Application Note

Food Safety

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Abstract

This method was developed for the determination of melamine in milk powder and egg. Solid phase extraction (SPE) and HPLC/UV are used consistent with the Chinese regulatory method. The sample preparation is performed using a polymeric mixed mode strong cation exchange resin. The separation and detection are performed by HPLC/UV. The limit of detection is 10 µg/kg. Linear calibration curves were obtained over the calibration range of 1 to 20 mg/kg. Overall recoveries range from 84 to 96 percent, with RSD values below 3.0 percent.

Agilent Technologies

Introduction

Melamine made headlines in September 2008 because it was found to be the contaminant responsible for the deaths of several infants and making many more sick. Melamine is used as an adulterant in milk and milk products because it causes a false positive value in a protein content measurement. The presence of melamine in foods is strictly regulated worldwide. Because milk is used in many other products for human and animal consumption, melamine is now detected in other food products in China and many other countries. The analysis for melamine is proceeding in many labs all over the world.

The Chinese government released a government regulation (GB/T 22388-2008) that established analytical methods for melamine in raw milk and dairy products and set a maximum residue limit (MRL) for melamine in food of 2 mg/kg. This application note describes the implementation and optimization of the solid phase extraction (SPE) method used for milk powder and egg described in GB/T 22388-2008.

Experimental

Reagents and Chemicals

Melamine was obtained from Sigma-Aldrich (Shanghai, China). A 1 mg/kg melamine stock solution was prepared in methanol and kept in the freezer (–4 °C). Appropriate dilutions using mobile phase generated working solutions that were prepared fresh daily.

Sample Extraction for Milk Powder or Egg

For egg, homogenize, place in a clean, sealed container, and store in the freezer at –4 °C.

For homogenized egg and milk powder, weigh 2.0 ± 0.01 g of sample and add to a 50-mL centrifuge tube. Add 15 mL of 1% trichloroacetic acid in water and 5 mL of acetonitrile, then cap. Sonicate for 10 minutes and then place samples on a vertical shaker for 10 minutes. Centrifuge the samples for 10 minutes at 4,000 rpm. Wet a filter paper with 1% trichloroacetic acid in water, then filter the supernatant into a 25.0-mL volumetric flask and bring to volume with 1% trichloroacetic acid in water. Transfer a 5.0 mL aliquot of the extract into a glass tube, and then add 5.0 mL purified water. Mix thoroughly on a vortex mixer.

SPE Purification

Agilent SampliQ SCX SPE cartridges (p/n 5982-3236, 3 mL, 60 mg) were used to clean up sample extracts. All SPE steps, including conditioning, sample load, wash, and the final elution, are performed without vacuum with a flow rate between 0.5 and 1 mL/min. For the drying step, vacuum is applied to quickly dry the cartridges. The procedure used for the SPE extraction is shown in Figure 1. Cartridges were conditioned with 3 mL of methanol, then 5 mL of water. A 10-mL prepared sample solution (equivalent to a 0.4-g sample) was passed through the cartridge. After the sample effused completely, the cartridge was washed with 3 mL of water and 3 mL of methanol. The entire effluent was discarded. The cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, the cartridge was eluted with 6 mL of 5% ammonium hydroxide in methanol. The eluent was collected and dried under nitrogen at 45 °C. The resulting residue was resuspended and made to a constant volume of 1 mL using the mobile phase. Then the residue was filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed.

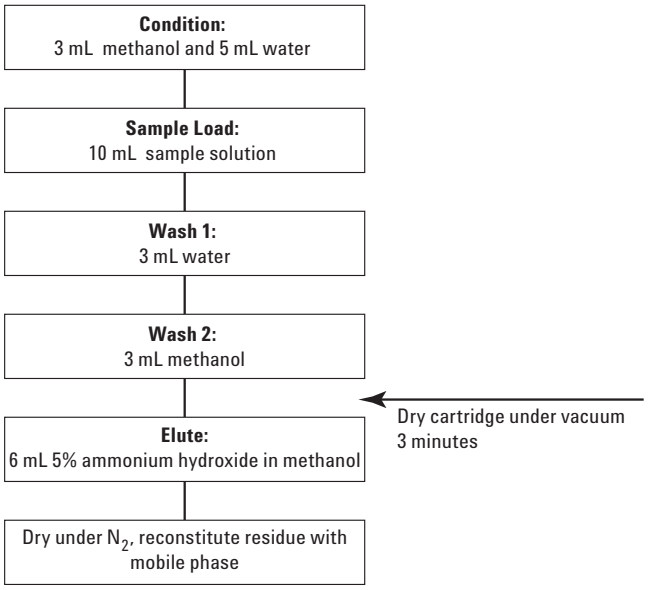

Figure 1. Melamine SPE procedure.

HPLC Conditions

Samples were analyzed on an Agilent 1200 Series HPLC with a diode array detector.

Column:	Agilent ZORBAX SB-C8 5 µm, 250 mm × 4.6 mm id (p/n 880975-906)
Flow rate:	1.0 mL/min
Column temperature:	40 °C
Detector wavelength:	240 nm
Injection volume:	20 µL
Mobile phase:	Acetonitrile-Buffer (15:85)
Buffer:	10 mmol/L citric acid and 10 mmol/L sodium octanesulfonate solution with a pH 3.0
Chromatography:	Isocratic

Results and Discussion

Linearity, Limit of Detection

Working standards were prepared at concentrations of 1, 5, 10, and 20 mg/kg by dilution of the stock solution with mobile phase. Linear regressions were calculated for melamine using the areas and the solution concentrations. The limit of detection (LOD) was the injection concentration whose signal-to-noise ratio was between 2 and 3. The linear range was between 1 and 20 mg/kg. The linearity and LOD results are shown in Table 1. The analysis of matrix blanks spiked with melamine shows no difference in area compared to solution based standards.

Table 1. Linearity and LOD of Melamine

Compound	Regression equation	Correlation coefficient	LOD (µg/kg)
Melamine	Y = 77.4698x + 0.2117	0.9999	10

Recovery and Reproducibility

The precision of the method is expressed as recovery of spiked melamine standard in milk or egg at concentrations of 2, 5, and 10 mg/kg levels. The analysis was performed in replicates of six at each level. Concentrations in the samples were calculated based on the external standard calibration curves. The chromatograms of the blank and spiked standard (2 mg/kg) are shown in Figures 2 to 5. The recovery and reproducibility data are shown in Table 2.

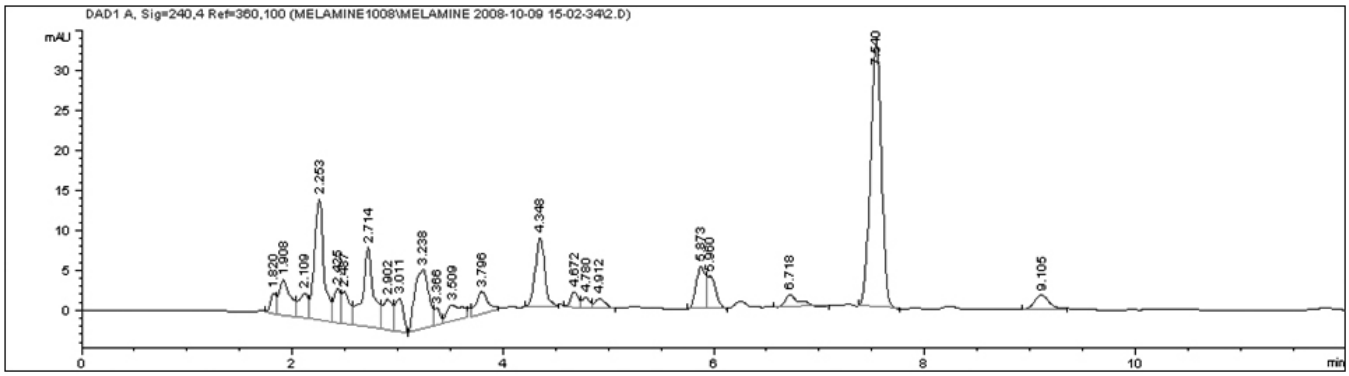

Figure 2. Chromatogram of a milk powder blank.

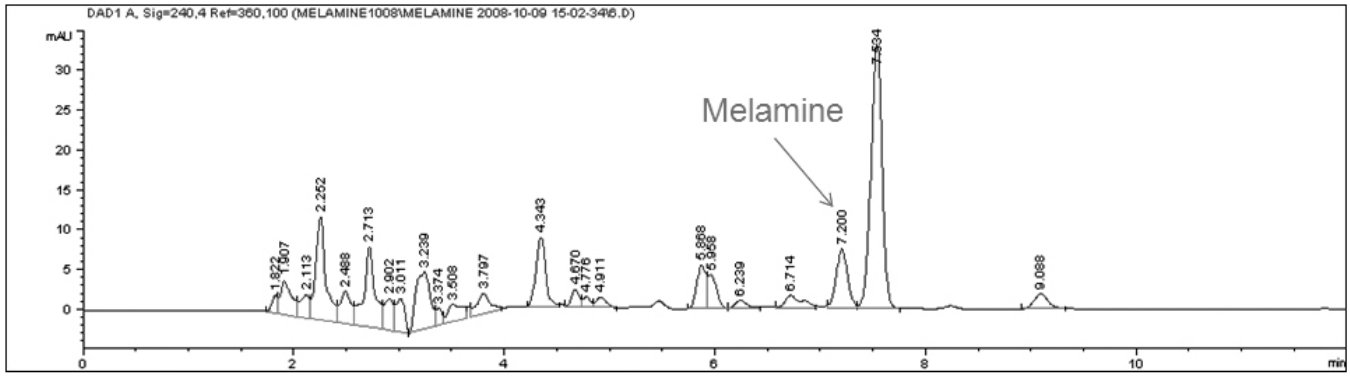

Figure 3. Chromatogram of a milk powder sample spiked at 2 mg/kg.

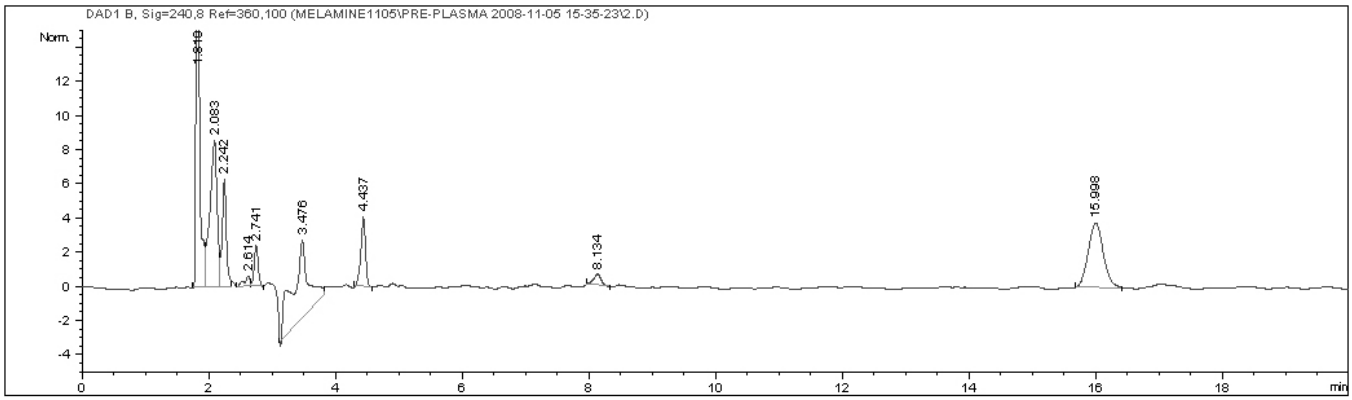

Figure 4. Chromatogram of an egg blank.

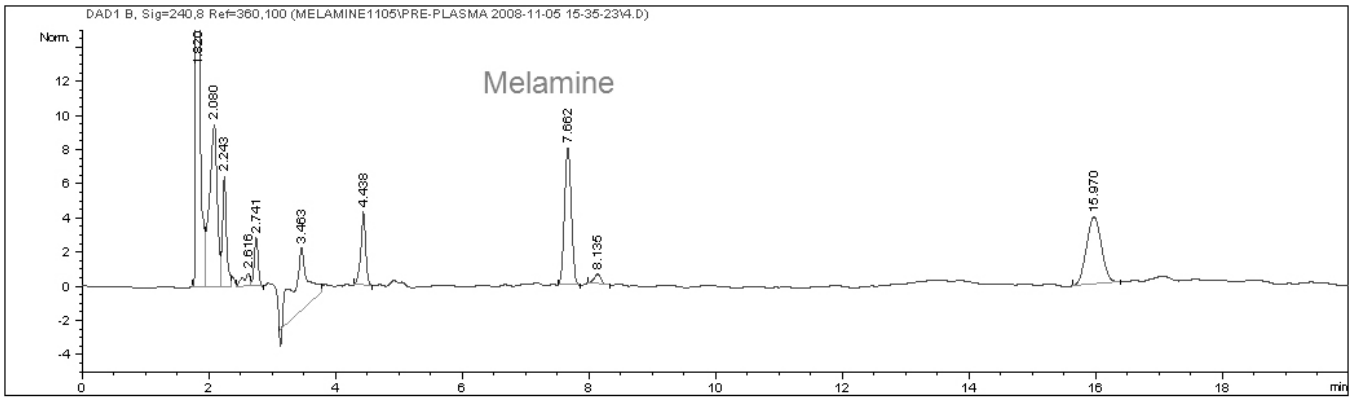

Figure 5. Chromatogram of an egg sample spiked at 2 mg/kg.

Table 2. Recoveries and Relative Standard Deviations of Melamine in Milk Powder and Egg Using Agilent SampliQ SCX SPE

Compound	Sample	Spiked level (mg/kg)	Recovery (%)	RSD (%)
Melamine	Milk powder	2	84.5	2.83
		5	85.3	2.56
		10	86.7	1.18
	Egg	2	95.2	3.00
		5	93.0	2.01
		10	95.7	2.89

Conclusions

Agilent SampliQ SCX solid phase extraction provides an effective single cartridge method for the purification and enrichment of melamine in milk powder and egg. The method is simple and complements any analytical procedure, such as LC/MS/MS or GC/MS. The recovery and reproducibility results, based on solution standards, are acceptable for melamine residue determination in milk powder and egg under the Chinese regulation. The impurities from milk and egg were minimal and did not interfere with the detection of melamine. The LOD (0.01 mg/kg) of melamine was significantly lower than the MRL (2 mg/kg).

Reference

GB/T 22388-2008, Determination of Melamine in Raw Milk and Dairy Products

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Agilent Technologies

Quantitative Liquid Chromatography Analysis of Melamine in Dairy Products Using Agilent's 1120 Compact LC and 1200 Rapid Resolution LC

Application Note

Food Safety

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Abstract

In this application, three different LC methods are developed for the determination of melamine in dairy products. The first is a modification of the U.S. FDA method [1]. An Agilent LC system (1120 or 1200) is used with a ZORBAX SB-C8 LC column to run in reversed-phase ion-pair mode for routine quantitation of melamine. The second method is targeted for high throughput using an Agilent Rapid Resolution LC (RRLC) system (1200SL) to speed melamine analysis by more than three times with a Rapid Resolution High Throughput (RRHT) column. The third is an alternative ion-exchange LC method where a ZORBAX 300SCX column is employed to successfully retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent. Due to the complexity of dairy product matrices, a cleanup step using solid phase extraction (SPE) is required for the above methods. The Agilent SampliQ SCX, a mixed-mode polymer SPE cartridge with combined reversed-phase and strong cation exchange properties, is used to successfully remove matrix interferences.

Agilent Technologies

Introduction

In March 2007, imported pet food ingredients contaminated with melamine caused renal failure in dogs and cats across the United States. Once again, this compound is in the news as an illicit adulterant in milk and milk products. The same contaminant is now being detected in other food products that contain milk imported from China and as global concern rises, widespread testing for melamine is proceeding.

The published analytical approaches include LC, LC/MS, and GC/MS. The LC method is being used for quantitative analyses of melamine. Liquid chromatographic separation of this small polar compound can be achieved by reversed-phase ion-pair liquid chromatography. The U.S. FDA developed this methodology for melamine in pet food in 2007. With a slight modification of the proportion of mobile phase, the method can be successfully applied to separate melamine from a variety of dairy product matrices.

The disadvantage of conventional HPLC is time and solvent consumption. The Agilent 1200 Series Rapid Resolution LC system is designed for highest throughput without loss of resolution or with better resolution in combination with the Agilent ZORBAX RRHT columns. In this application note, the conventional HPLC method is transferred from a 4.6 mm × 250 mm, 5 µm ZORBAX SB-C8 column to a 4.6 mm × 50 mm, 1.8 µm RRHT ZORBAX SB-C8 column with equivalent results, and the LC run time is shortened from almost 20 minutes to 6 minutes.

An alternative approach for liquid chromatographic separation of this small polar compound is ion exchange chromatography. Agilent ZORBAX 300SCX is an ionic bonded-phase column packing used for cation exchange high-performance liquid chromatography. This packing consists of an aromatic sulfonic acid moiety covalently bonded to ZORBAX porous silica. This column is successfully applied to retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent.

For complex dairy product matrices, it is necessary to remove interferences such as protein, sugar, and fat before LC injection. Solid-phase extraction (SPE) is a simple way to clean up the complex matrix extract. SampliQ is a new family of SPE cartridges from Agilent with a wide range of sorbent chemistries. Among this family, the mixed-mode SampliQ Strong Cation Exchange (SCX) cartridge is a sulfonic acid-modified divinyl benzene polymer with both ion exchange and reversed-phase retention properties. This makes the SampliQ SCX very effective for cleanup after solvent extraction.

Experimental

Standard Preparation

A stock solution of melamine at 1,000 µg/mL is prepared in methanol by sonication. Dilutions in mobile phase are made up at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 µg/mL concentrations.

Sample Preparation

The sample preparation process is a modification of the China national standard [2].

Sample Extraction Procedure

For liquid milk, milk powder, yogurt, ice cream, and creamy candy samples:

- Weigh 2 ± 0.01 g of sample and add to a 50-mL centrifuge tube, add 15 mL of 5% trichloroacetic acid in water and 5 mL of acetonitrile, then cap.
- Sonicate for 10 min and then place samples on vertical shaker for 10 min. Centrifuge for 10 min at 4000 rpm.
- Wet filter paper with 5% trichloroacetic acid in water, then filter the supernatant into a 25.0-mL volumetric flask, and bring to volume with 5% trichloroacetic acid in water.
- Transfer a 5.0-mL aliquot of the extract into a glass tube, and then add 5.0 mL purified water. Vortex to mix thoroughly.

For cheese, cream, and chocolate samples:

- Weigh 2 ± 0.01 g of sample, grind with 8~12 g of sea sand in a mortar, and then transfer into a 50-mL centrifuge tube.
- Wash the used mortar with 5 mL of 5% trichloroacetic acid in water three times, transfer washings into a 50-mL centrifuge tube, and then add 5 mL of acetonitrile.
- Proceed with the sonication and other steps as described in the previous preocedure..
- If the sample is very fatty, defat the extract using liquid-liquid extraction with hexane saturated with 5% trichloroacetic acid in water before cleanup by SPE.

Sample Cleanup Procedure

A SampliQ SCX SPE cartridge (p/n 5982-3236, 3 mL, 60 mg, or p/n 5982-3267, 6 mL, 150 mg) can be used to clean up sample extracts; the latter is used in this application note. All SPE elution steps, including conditioning, sample load, washing, and the final elution, are performed with a flow rate of less than 1 mL/min except for drying the cartridge by applying vacuum.

- Condition the SPE cartridge with 5 mL of methanol followed by 6 mL of water.
- Load the above sample extract to the conditioned cartridge. Wash the cartridge with 5 mL of water followed by 5 mL of methanol.
- Dry the cartridge by applying vacuum, and then elute with 5 mL of 5% ammonium hydroxide in methanol.
- Evaporate the eluate to dryness under a stream of nitrogen at approximately 50 °C.
- Reconstitute the dried extract in 1.0 mL of mobile phase, vortex for 1 min, and filter through a 0.2-µm regenerated cellulose membrane filter (p/n 5064-8222) into a glass LC vial.

Instrumentation and Conditions

Conventional HPLC method using 1120 Compact LC or 1200 LC:

- Agilent 1120 Compact LC system with gradient pump (degasser inside), autosampler, column compartment, and variable wavelength detector (VWD) or equivalent 1200 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 (also known as ZORBAX Rx-C8), 4.6 mm × 250 mm, 5 µm (p/n 880975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	20 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	20 min

High-Throughput Method Using 1200SL RRLC:

- Agilent 1200SL Series binary pump, degasser, wellplate sampler, thermostatted column compartment and diode array detector (DAD)
- ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 RRHT, 4.6 mm × 50 mm, 1.8 µm (p/n 827975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	8 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	6 min

Ion Exchange Chromatography Method with 1120 Compact LC or 1200 LC:

- Agilent 1200 Series binary pump, degasser, wellplate sampler, thermostatted column compartment and variable wavelength detector (VWD) or equivalent 1120 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX 300SCX, 4.6 mm × 150 mm, 5 µm (p/n 883952-704)
Buffer	50 mM ammonium formate solution, adjust to pH 3.0 with formic acid
Mobile phase	15:85 buffer:acetonitrile
Flow rate	1.0 mL/min
Injection volume	10 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	5.5 min

Results and Discussion

Separation of Melamine in Dairy Products by Reversed-Phase Ion-Pair LC

Melamine is not retained by reversed-phase LC and thus elutes with the solvent and unretained matrix interferences. However, using an ion-pairing reagent with reversed-phase chromatography, melamine can be well retained and separated from interferences. Figure 1 (a) is the chromatogram of melamine standard by reversed-phase ion-pair LC. Figure 1 (b) is the chromatogram of a positive yogurt sample after cleanup with the Agilent SampliQ SCX SPE cartridge.

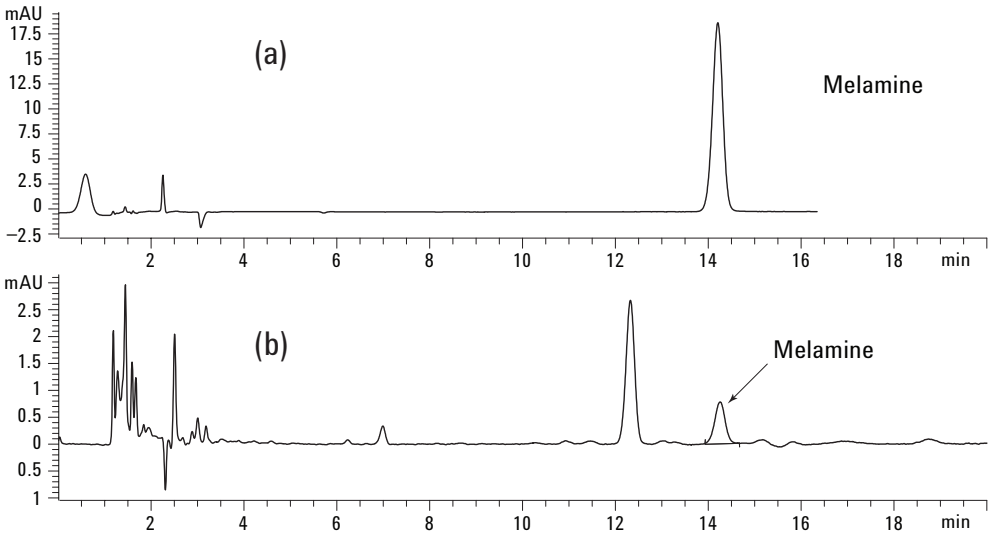

Figure 1. Separation of (a) 20 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 14.2 minutes.

High-Throughput Analysis by Agilent 1200SL RRLC with RRHT Column

With the Agilent 1200 Series RRLC system, high throughput is possible. In combination with the Agilent ZORBAX RRHT columns, excellent chromatographic resolution can be achieved at much shorter run times than with a conventional LC system. A RRLC method is developed to dramatically increase the sample throughput for the determination of melamine in dairy products. Figure 2 (a) is the chromatogram of a melamine standard by the RRLC method with the retention time of melamine at 2.8 minutes.

Figure 2 (b) is the chromatogram of the same yogurt sample in Figure 1 (b). In order to make sure that the column is clear for the next injection, the total run time is extended to 6 minutes. The high throughput RRLC method is applied in the variety of dairy products matrices, including yogurt, liquid milk, and milk powder to demonstrate that the same resolution is achieved as with the conventional HPLC method. The calibration curve for the RRLC method is shown in Figure 3. The calibration includes 0.8, 2.0, 20.0, 40.0, and 80.0 µg/mL. The instrumental LOD (limit of detection) of the RRLC method is 0.03 µg/mL.

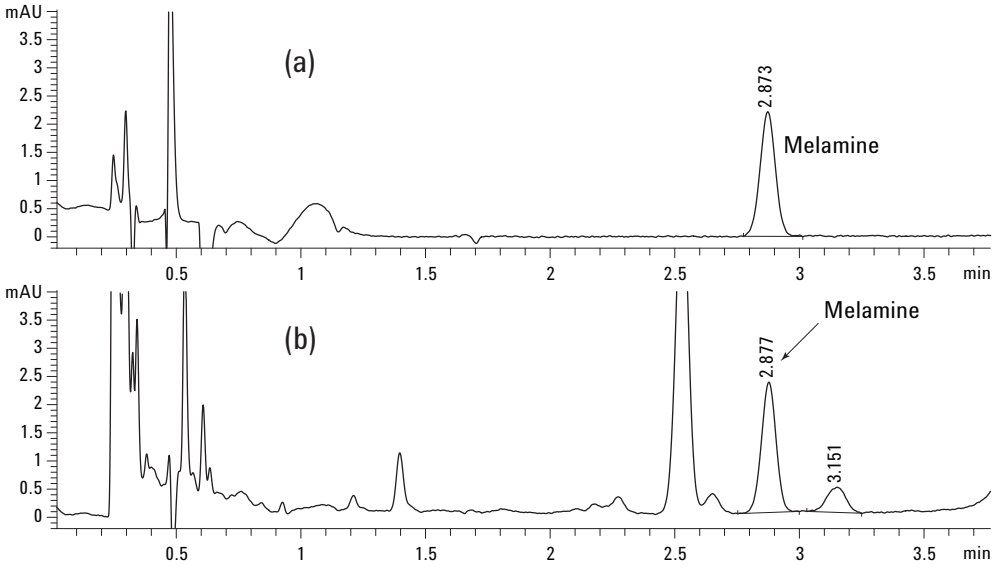

Figure 2. Separation of (a) 0.8 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 2.8 minutes.

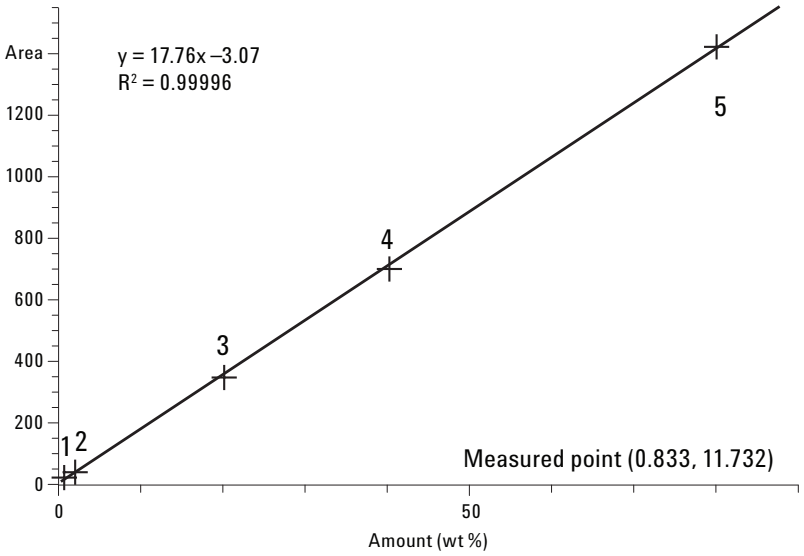

Figure 3. Calibration curve of RRLC method.

Ion Exchange Chromatographic Method

An alternative to ion-pair reversed-phase chromatography for melamine is ion exchange chromatography (IEC). The Agilent ZORBAX 300SCX is used for cation exchange high-performance liquid chromatography (HPLC). This column is employed to separate melamine in dairy product matrices with sufficient retention to separate matrix interferences. Figure 4 shows the separation of melamine from interferences without the SPE cleanup step. Generally, the total run time of the ion exchange chromatography is only 5.5 minutes with an LOD of 0.05 µg/mL, as shown in Figure 5. The calibration curve for the IEC method is shown in Figure 6. The calibration points include 0.5, 1.0, 5.0, 10, 50, and 100 µg/mL. Although the separation is shown to be interference free for raw milk and liquid milk without any additive, it is still recommended that the cleanup step be included to ensure robust methodology for running many samples and samples of different matrices.

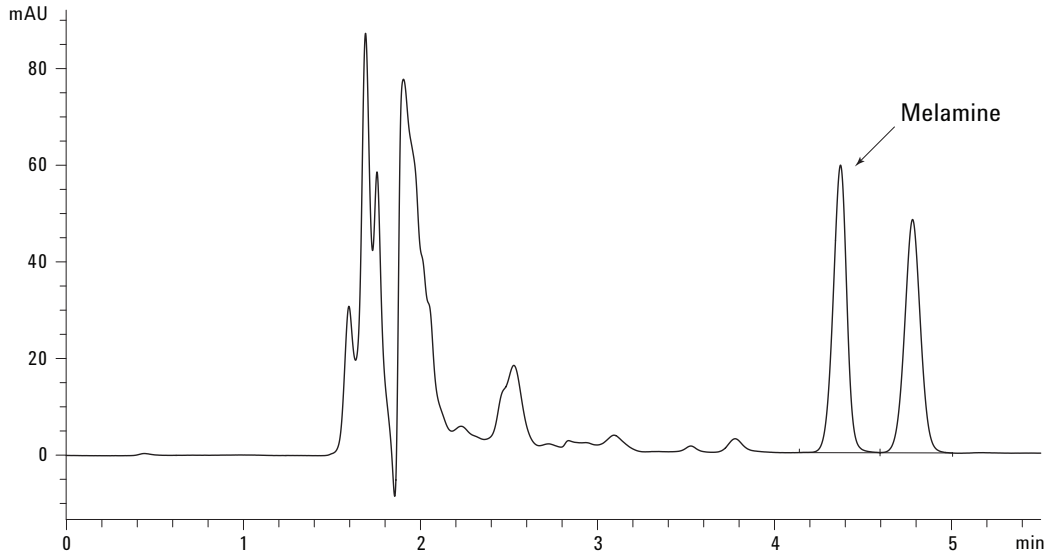

Figure 4. Separation of melamine in milk powder sample by IEC without cleanup by SPE.

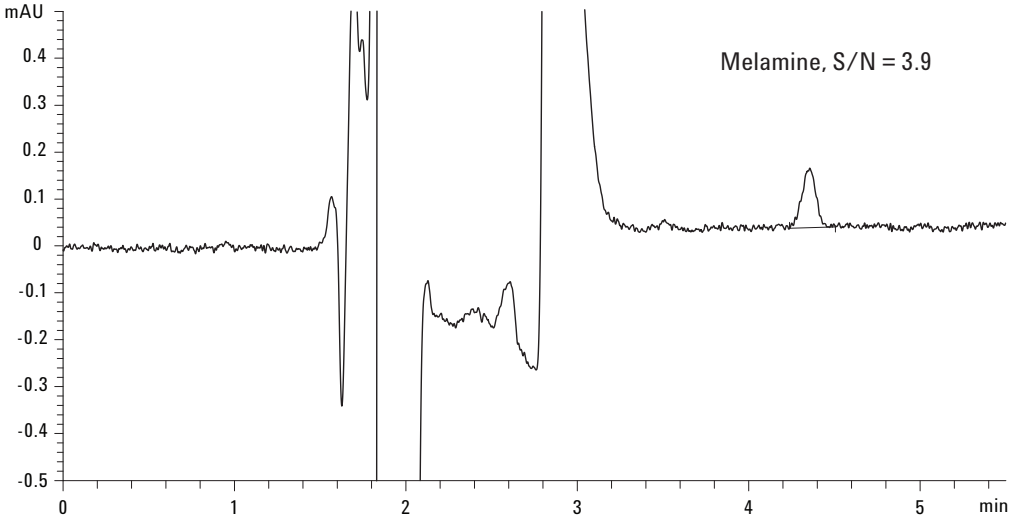

Figure 5. Limit of detection (LOD) for melamine at the concentration of 0.05 µg/mL.

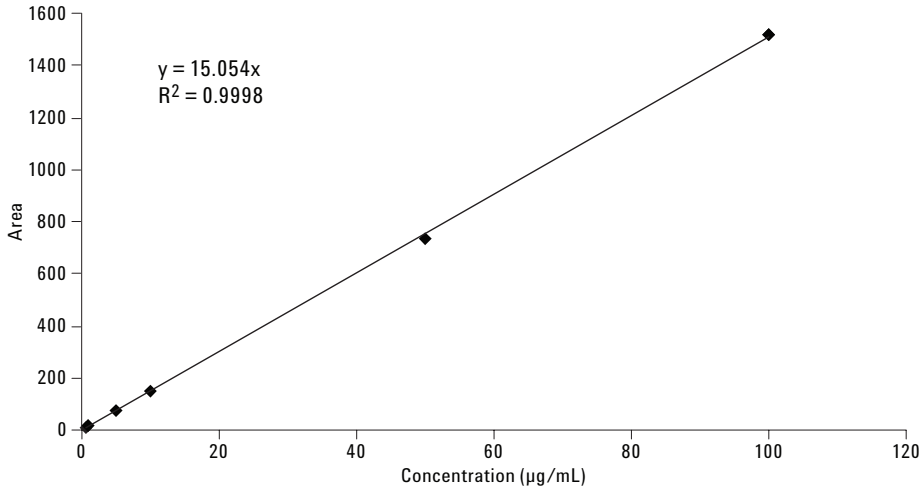

Figure 6. Calibration curve of IEC method.

Conclusions

Three approaches are described in this application note; the first is a reversed-phase ion-pair LC method employing Agilent 1120 compact LC or 1200 HPLC with an SB-C8 column. The second is a high-throughput method, which reduces the LC run time from 20 minutes to 6 minutes using the Agilent 1200 RRLC with the ZORBAX RRHT SB-C8 column. The last is an IEC method using the Agilent ZORBAX 300SCX column. Each successfully separates melamine from matrix interferences and provides identification by retention time and quantitative results. The results of this study, including sample cleanup with SampliQ SCX SPE cartridges and the three separation approaches, show that a complete solution from Agilent for the determination of melamine in dairy products is provided. The reversed-phase ion-pair method is based on the FDA and China national standards. However, the IEC method is simple, quick, sensitive, and robust. With this method, melamine can be successfully retained using a simple mobile phase without the presence of an ion-pair reagent.

References

1. Updated FCC Developmental Melamine Quantitation (HPLC-UV), April 2, 2007
2. GB/T 22388-2008 Determination of melamine in raw milk and dairy products, October 7, 2008

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Agilent Technologies

A Total Solution for the Analysis of Melamine and Cyanuric Acid in Pet Food by GC/MS and Aqueous Normal-Phase LC/MS/MS

Application

Food

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Abstract

The Agilent 7890A-5975C GC/MS and 1200SL-6410 Triple Quadrupole LC/MS are used to analyze melamine and cyanuric acid in pet food and related raw material. The GC/MS method employs extraction and then trimethylsilyl derivatization and is used here for screening. The detection limit is 10 µg/g. The LC/MS/MS method requires only extraction and then a simple isocratic elution on a normal-phase silica column (Rx-Sil) with water and acetonitrile mobile phase containing 5 mM ammonium acetate. The separation of the two compounds takes 5 min. The linear range is from 50 pg/mL to 50 ng/mL for melamine and from 1 ng/mL to 100 ng/mL for cyanuric acid. The LC/MS/MS method is demonstrated by analysis of the target compounds in complex food matrix.

Introduction

The illicit additives melamine and cyanuric acid in pet food have caused the death of cats and dogs. This resulted in the U.S. FDA recalling millions of packages of pet food in the past year. Consequently, it became a hot issue to determine the content of melamine and cyanuric acid in pet food and related raw materials worldwide.

A screening method using GC/MS for both these compounds was published by the U.S. FDA [1]. According to this procedure, the detection limit for GC/MS screening method is 10 µg/g using a trimethylsilyl (TMS) derivatization process before injection. Although this method is used for screening, it could be used as well for confirmation and quantitation using the SIM/SCAN function of the Agilent 5975. In comparison with the GC/MS method, the LC/MS/MS method presented here simplifies the sample preparation process without derivatization and provides confirmation and quantitation in one step. Additionally, the high sensitivity and selectivity of LC/MS/MS could cover trace-level analysis in animal fluids and tissue to high levels in food and food ingredients.

Experimental

Sample Preparation

Sample preparation for GC/MS was done as per the FDA method [1]. To a 0.5-g sample, a mixed solvent (10:40:50 diethylamine:water:acetonitrile) is added and mixed well to thoroughly wet the entire sample. The mixture is then sonicated for 10 min, centrifuged at 5000 rpm for 10 min, and then filtered into a vial using a 0.45 µm nylon disc. Derivatization is done by transferring 200 µL of the extract to an autosampler vial and evaporating to dryness with a low flow of dry nitrogen at 70 °C. To the dried extract is added 200 µL pyri-

dine and 200 µL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture is vortexed and allowed to incubate at 70 °C for 45 min.

Sample preparation for LC/MS/MS was done by grinding about 5 g sample by a mill and mixing thoroughly. To a 0.5-g sample, 20 mL of acetonitrile/ water (50:50) is added, capped, and vortexed. The solution is sonicated for 30 minutes and then filtered with a 0.20-µm PTFE syringe filter (Agilent P/N 5185-5843). If the concentration of the target compounds is beyond the linear range of the method, further dilution of the extract with acetonitrile:water should be made. Note that if cyanuric acid and melamine are in the same solution at high concentrations, 10:40:50 diethylamine: water:acetonitrile is needed to disrupt the ion pair.

GC/MS Method Details

Instrument:	7890A GC with 5975C MSD
Software:	MSD ChemStation E.01.00 with NIST 05a MS Library 2.0d

GC Conditions

Column:	DB-5MS 30 m × 0.25 mm × 0.25 µm (P/N: 122-5532)
Inlet temperature:	290 °C
Injection mode:	Splitless or split (1:20)
Injection volume:	1 µL
Carrier gas flow:	He (constant flow) at 1.3 mL/min
Oven program:	75 °C (hold 1 minute) to 300 °C at 30 °C /minute (hold 2 min)
Total run time:	10.5 min.
Transfer line temperature:	280 °C

MS Conditions (SIM/Scan Mode)

Tune:	Autotune
Acquisition mode:	EI; SIM/scan mode
Solvent delay:	3.5 minutes
MS temperature:	230 °C (Source); 150 °C (Quad)

Scan Parameters

Scan range:	40–450 amu
Sampling rate:	2 (scan rate at ~3.6 scans/sec)
Threshold:	100

SIM Parameters

GROUP 1

Group identification:	Auto_1							
Resolution:	Low							
Plot 1 Ion:	345.10							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell		
	188.00	25	330.10	25	345.10	25		

GROUP 2

Group identification:	Auto_2							
Resolution:	Low							
Group start time:	6.76							
Plot 1 ion:	327.20							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell	Mass	Dwell
	197.00	25	285.10	25	327.20	25	342.20	25

LC/MS Method Details

Instrument:	1200 SL RRLC with 6410 Triple Quad MS
Software:	MassHunter B.01.03

LC Conditions

Column:	ZORBAX Rx-Sil, 2.1 mm × 150 mm, 5 µm (P/N 883700-901)
Column temperature:	40 °C
Mobile phase:	A = 5 mM ammonium acetate in water B = 5 mM ammonium acetate in acetonitrile
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Isocratic:	95% B
Stop time:	5.5 minutes
Needle wash:	50:50 acetonitrile/water; flush port 10 seconds

MS Conditions

Ion source:	ESI
Polarity:	Positive and negative
Nebulizer gas:	Nitrogen
Ion spray voltage:	4000 V
Source temperature:	350 °C
Resolution:	Q1 (unit) Q3 (unit)
Scan mode:	Multiple reaction monitoring (MRM)
Segment:	Segment 1 = 0~2 min negative mode for cyanuric acid Segment 2 = 2~5.5 min positive mode for melamine
Delta EMV:	600 V

Parameters of multiple reaction monitoring (MRM) are shown in Table 1.

Table 1. MRM Parameters for Cyanuric Acid and Melamine

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
1.45	Cyanuric acid	128	42	200	100	30
		128	85	200	100	5
4.92	Melamine	127	85	200	100	20
		127	68	200	100	35

Results and Discussion

The Selection of Monitoring Ions for SIM Mode in GC/MS

Selective ion monitoring (SIM) is often used to improve the detection limit and quantitative reproducibility. With SIM mode, the MS monitors only a few ions for each target compound within the retention time (RT) range that the target elutes from the column. By monitoring only a few specific ions, the signal-to-noise ratio (S/N) improves significantly. Therefore, the selection of the ions monitored for SIM mode is very important for good analytical result. Usually, a few ions of highest abundance are chosen for SIM to maximize the response of the target compounds. However, if the interference from the matrix is significant, the most characteristic ions should be chosen to minimize signal disturbance even if their abundance is relatively lower. In this application, the influence of ion selection was demonstrated in Figure 1. With the use of more characteristic ions, the chromatogram has much less interference than the chromatogram with the highest abundance ions. The use of SIM/SCAN allows both the highest sensitivity and reproducibility with the additional benefit of full identification contained in the complete spectra of the analytes.

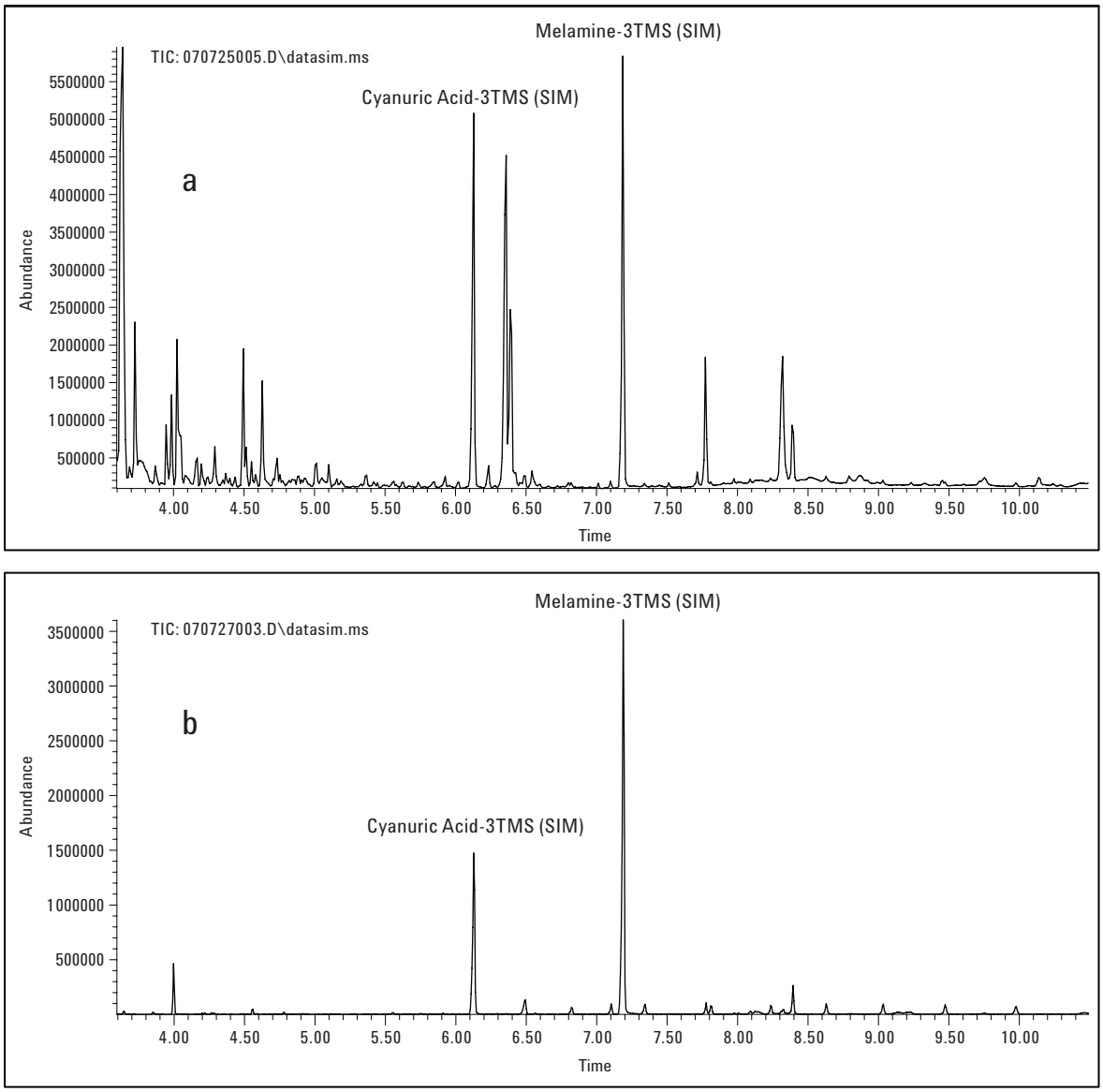

Figure 1. The influence of the selection of the ions monitored in SIM mode shown with spiked dry cat food; a) the ions of highest abundance were used (cyanuric acid: *m/z* 73, 147, 171; melamine: *m/z* 327, 330, 342, 345) and b) characteristic ions were used (cyanuric acid: *m/z* 188, 330, 345; melamine: *m/z* 197, 285, 327, 342).

The Retention of Melamine and Cyanuric Acid on a Normal-Phase Column

Liquid chromatographic separation of small highly polar compounds like melamine and cyanuric acid can be achieved by retention mechanisms such as ion-exchange and ion-pair reversed-phase liquid chromatography (IP-RPLC). In general, the ion-exchange approach is not very suited to the electrospray interface (ESI) due to the use of buffers with high ionic strength. Likewise, volatile ion-pair reagents can provide satisfactory results, but again are not well suited for routine use with LC/MS. In addition, ion pair separation of both an acid and a base in the same analysis is difficult.

A much more suitable approach for these compounds is known as aqueous normal-phase chromatography or hydrophilic interaction chromatography (HILIC) [2]. This mode of separation is defined as a partition of a polar analyte between a relatively nonpolar mobile phase and a polar semi-immobilized liquid phase associated with the stationary phase. Normal phase chromatography is usually associated with adsorption of the analyte on the stationary phase. It is believed that in this mode water is trapped on the surface of the stationary phase and evokes partitioning instead of adsorption. Both cyanuric acid and melamine can be successfully retained by the Rx-Sil column with 1.45 minutes and 4.92 minutes of retention time, respectively, under these conditions.

Quantitative Analysis of Melamine and Cyanuric Acid in Different Matrices

A time segment program was used to switch between the negative mode for cyanuric acid and the positive mode for melamine. Linearity of the developed method was studied by three replicate injections at each concentration level. The calibration curves of melamine and cyanuric acid are displayed in Figure 2. The linear range of the method is 50 pg/mL to 50 ng/mL for melamine and 1 ng/mL to 100 ng/mL for cyanuric acid. The detection limits for melamine and cyanuric acid are shown in Figure 3. The S/N ratio (peak-to-peak) is 12.7 for 50 pg/mL melamine and 12.8 for 1 ng/mL cyanuric acid.

Pet food, wheat gluten, corn meal, and rice protein samples were extracted and analyzed for both target compounds. Examples of a spiked wheat gluten sample are given in Figure 4. A mixture of acetonitrile and water (50:50) is most efficient for the extraction of melamine and cyanuric acid from the different matrices. When injecting 10 µL of sample, the extract should be diluted to 5% or less water or the peaks will be distorted. Otherwise, reducing the injection volume to 1 µL is the easiest way to eliminate the distortion.

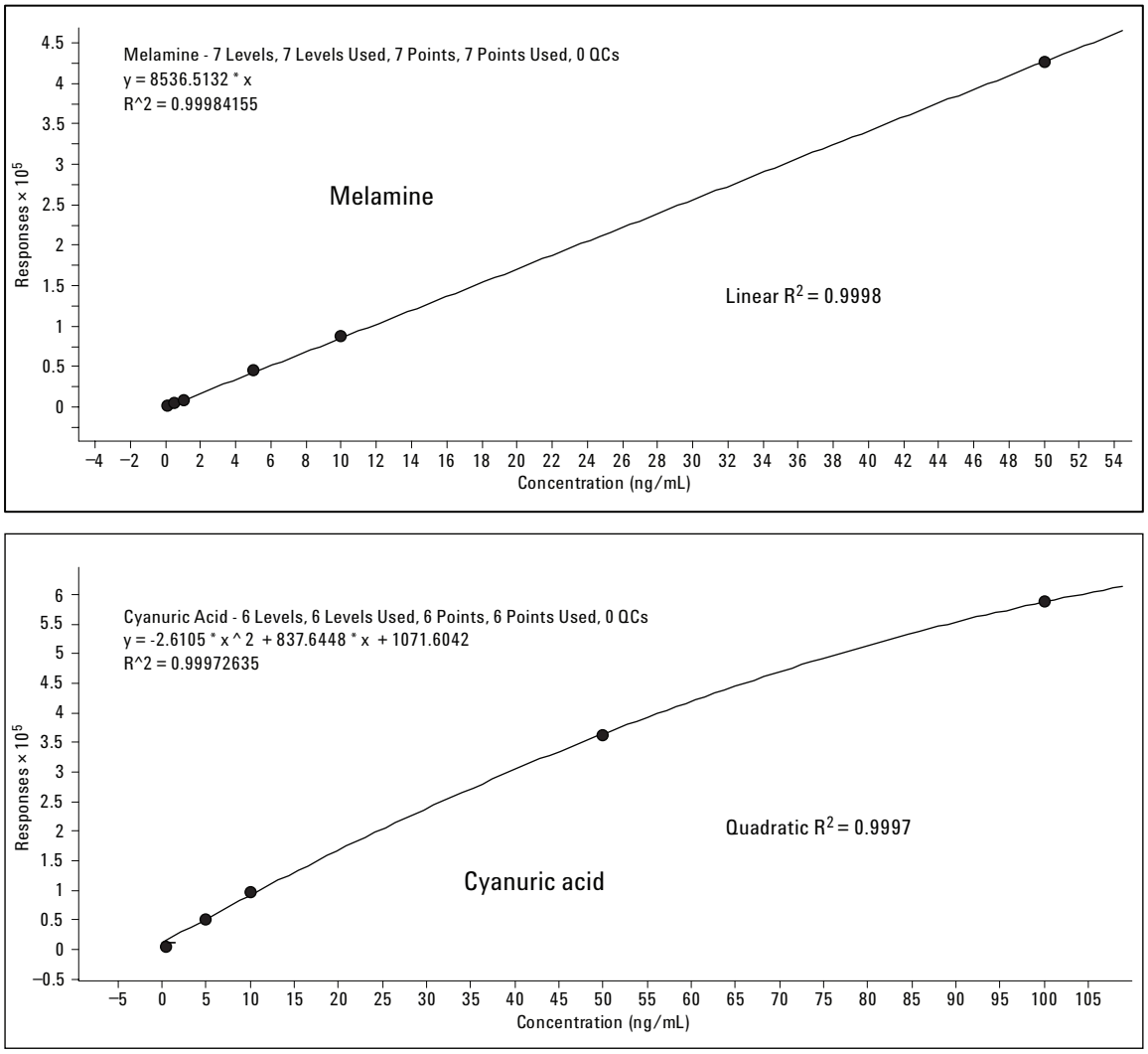

Figure 2. Linearity of melamine and cyanuric acid.

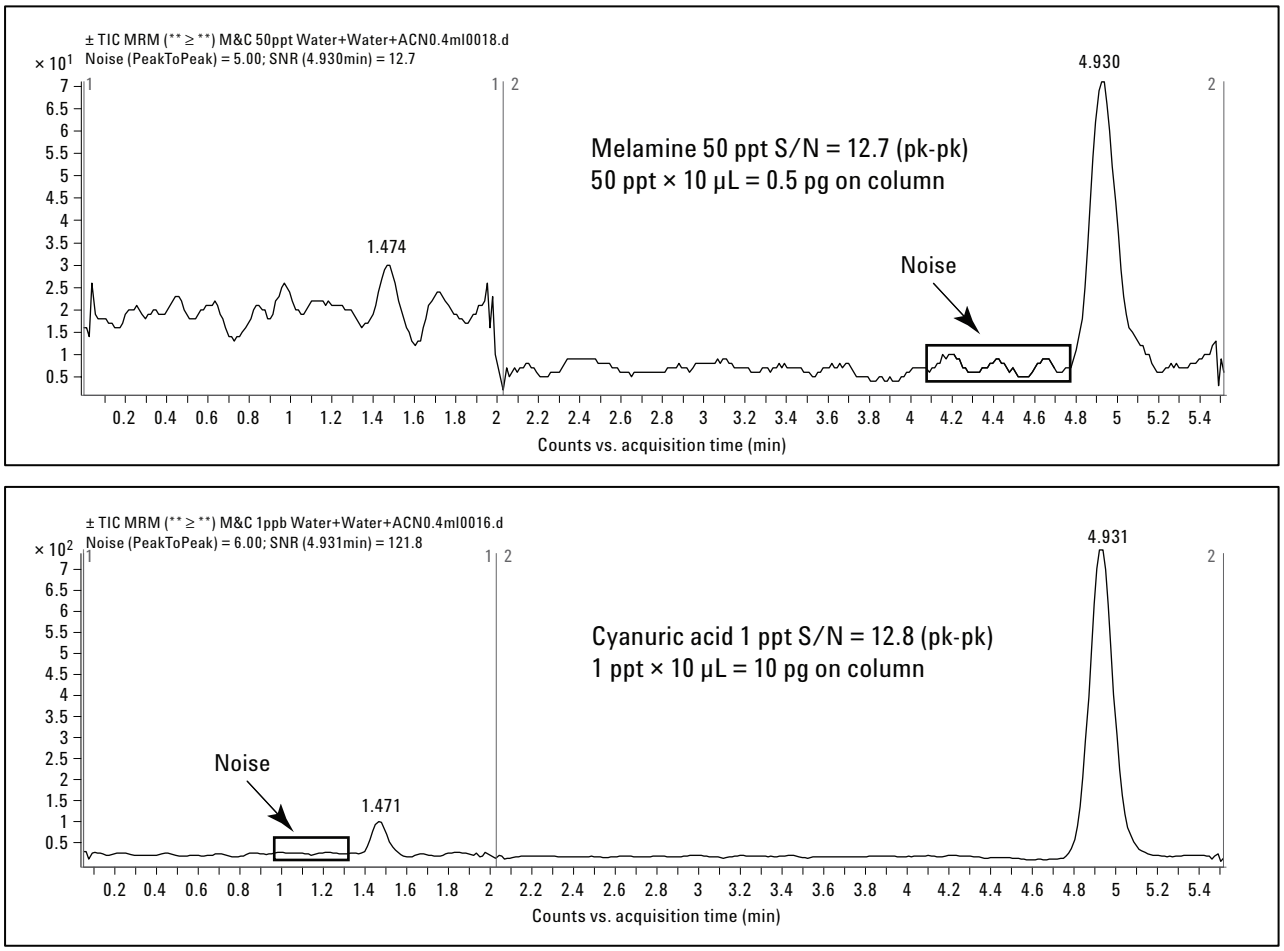

Figure 3. Detection limits for melamine and cyanuric acid.

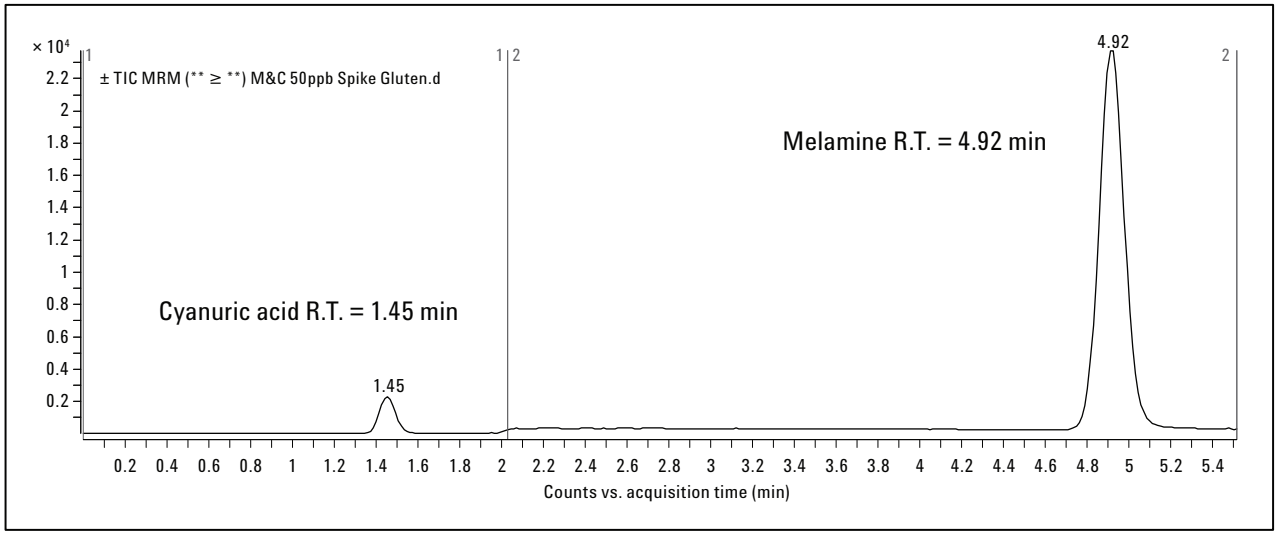

Figure 4. Total reaction monitoring chromatogram of spiked wheat gluten sample.

Conclusions

This application demonstrates that GC/MS or LC/MS/MS methods can readily be used for the analysis of melamine and cyanuric acid in pet food and its ingredients. In the example shown, the GC/MS method was used to screen the presence of melamine and cyanuric acid in pet food using SIM mode. However, the method can be used for quantitation and confirmation using SIM/SCAN so long as very low detection levels are not required. In addition, an LC/MS/MS method has been shown with both a simple sample preparation procedure and an aqueous normal-phase separation. The method is highly sensitive and selective and is used both for the confirmation and quantitation of melamine and cyanuric acid in pet food and related raw materials. The detection limits of 50 pg/mL for melamine and 1 ng/mL for cyanuric acid would readily allow for the analysis of these compounds at trace levels in animal fluids and tissues.

References

1. GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric Acid, Version 2, May 7, 2007. http://www.fda.gov/cvm/MelaminePresence.htm
2. K. I. Petrus Hemström, “Hydrophilic interaction chromatography.” *Journal of Separation Science* **2006**, 29, (12), 1784-1821.

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Agilent Technologies

Rapid Screening of Melamine and Cyanuric Acid in Milk Products Using Agilent J&W HP-5ms GC Column and Agilent 7890A/5975C GC/MSD with Column Backflushing

Application Note

Food Safety

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Abstract

A rapid screening method for the determination of melamine and cyanuric acid in milk products is developed using Agilent 7890A/5975C GC/MSD along with Agilent J&W HP-5ms GC columns. With the backflushing by capillary flow technologies, this method eliminates the time consumption for column bakeout after elution of target compounds, so as to significantly shorten the GC run time from more than 70 minutes to 14.5 minutes. Good linearity was obtained within the range of 10 to 200 µg/g, with correlation coefficients greater than 0.9996. The recoveries of both target compounds are greater than 95%.

Agilent Technologies

Introduction

The contamination of food with melamine and cyanuric acid has attracted much attention all over the world. For the analysis of milk products, one of the major problems is the presence of less-volatile and nonvolatile matrix components, such as protein and fat. These components might contaminate the analytical system if the sample is introduced without selective sample preparation. The usual way to remove the matrix is to bake the column at high temperature, which often takes much longer than the sample run time for the analysis of interest. For example, the application of a milk extract of FDA method [1] usually takes about 70 minutes. Selective extraction or selective sample introduction is not easy, since the target compounds cover a broad volatility and polarity range. Moreover, for a routine QC analysis, laboratories want to reduce the typical cycle time by 25 to 60 minutes to improve productivity. The Agilent 7890A/5975C GC/MSD system meets this demand by using backflushing and faster cooling.

In this application note, a modified U.S. FDA method is developed using a standard split/splitless inlet and Agilent Capillary Flow Technology. A three-way splitter device with a makeup gas is placed at the end of the column and connected to MSD with a restrictor, thereby allowing column outlet pressure to be controlled with auxiliary electronic pneumatic control (EPC). By decreasing the column inlet pressure and increasing the column outlet pressure after the last peak of interest eluted from the column, the column flow is reversed, and the matrix interference, especially high boiler, can be removed out of the inlet end of the column. [2]

Experimental

Standards and Reagents

The standards and reagents used in the experiment are listed in Table 1. Stock solutions of melamine and cyanuric acid, each at a concentration of 1,000 µg/mL, were separately prepared in methanol. Stock solutions of standards are stored in the refrigerator.

Table 1. Standards and Reagents

Standard	Melamine	Sigma-Aldrich	>99% purity
	Cyanuric acid	Sigma-Aldrich	>99% purity
Solvent	Methanol	Fisher Scientific	HPLC grade
	Pyridine	Fisher Scientific	Certified A.C.S. Reagent
Silylating reagent	BSTFA with 1% TMCS*	Supelco	/

* BSTFA: bis(trimethylsilyl)trifluoroacetamide, TMCS: Trimethylchlorosilane

Instrument

The experiment was performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet, an Agilent 5975C GC/MSD with Triple-Axis Detector, and an Agilent 7683B automatic liquid sampler (ALS). The split/splitless inlet is fitted with a long-lifetime septum (P/N 5183-4761) and split injection liner (P/N 5188-4647). Injections are made using a 10-µL syringe (P/N 9301-0714). The instrument conditions are listed in Table 2.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Column:	HP-5ms, 30 m × 0.25 mm × 0.25 µm (P/N 19091S-433)
Inlet temperature	EPC, split/splitless @ 250 °C
Injection volume	1 µL, split ratio at 3:1
Carrier gas	Helium, constant flow mode, 1.3 mL/min
Oven program	75 °C (1 min), 30 °C/min to 300 °C (1 min)
Post-run	280 °C, hold for 5 min (backflushing duration)
Transfer line	280 °C
MS Conditions	
MS	EI, SIM/scan
Solvent delay	4.2 min
MS temperature	230 °C (source), 150 °C (quad)
Scan mode	Mass range (40 to 450 amu)
SIM mode	Ion (melamine: 342, 327*, 171, 99; cyanuric acid: 345*, 330, 188)
Backflush Conditions	
Device	3-way splitter (P/N G3183B)
Restrictor	0.706 m × 180 µm id
Outlet	PCM (P/N G1530-63309)
Outlet pressure	2 psi (60 psi for post-run)
Inlet pressure	2 psi (for post-run)

* Quantitative ion

Sample Preparation

Extraction

0.5 g of sample (powder or liquid) was weighed into a 20-mL polypropylene centrifuge tube; 5 mL of methanol was added. The sample was capped, vortex mixed, and then sonicated for 10 minutes. After the sample was centrifuged at 4,000 rpm for 6 minutes, the supernatant fluid was filtered through a 0.45-µm PTFE filter into a glass GC vial.

Derivatization

40 µL of the above extract was transferred into a glass GC vial. The extract was evaporated to dryness under a stream of nitrogen at approximately 70°C. 50 µL of pyridine and 50 µL of BSTFA were added. The sample was vortex mixed and incubated at 70 °C for 30 minutes.

Results and Discussion

7890A/5975C GC/MSD with a Backflush System for Milk Extracts

Milk extract usually contains many low-volatile or nonvolatile constituents. These compounds may stay near the front of the column until the oven temperature is high enough to move them through the column. In this application, a three-way splitter with makeup gas was employed to perform the backflush. The device has makeup gas supply tubing and four connectors, one connector for the analytical column and three connectors for the restrictor tube connecting to three available detectors. Since only an MSD is used as a detector in this application, the first two connectors were capped in the direction of makeup flow, the third connector was for the column in, and the last connector for the restrictor out to the MSD. This flow configuratin was used to avoid peak broadening due to improper flow sweeping. The length and internal diameter of the restrictor tubing is 0.706 m and 0.18 mm, respectively. The schematics of the GC/MS system configuration and tubing connection for the three-way splitter are shown in Figure 1.

First, a milk extract was analyzed in a typical mode – without backflush – by programming the oven to 300 °C to ensure that late eluters were eluted. It took more than 70 minutes to elute all the constituents in the extract (Figure 2A). Then, the extract was analyzed with a 5-minute backflush (Figure 2B). The backflush was accomplished by increasing the pressure in the outlet and decreasing the inlet pressure. The column flow was reversed to push the "heavy" constituents through the column inlet and out of the split vent. Figure 2C shows a 70-minute blank solvent run after the backflush. The blank run shows that the column was clean after backflushing, except for some peaks coming from the vial septa. Instead of baking the column at 300 °C for 55 minutes, the heavy matrix components were effectively removed from the column through backflushing. This reduced the run time from 70 minutes to 14.5 minutes, or a 4.8-fold increase in speed.

For a complex matrix, even baking for a long time cannot thoroughly remove the high boiler, which may result in peak retention time shift in the following injection. In Figure 3, two consecutive runs of the extract with 5-minute backflushing are shown. Excellent retention time and peak area repeatability were obtained, with no evidence of carryover, no emerging ghost peaks, and no increasing baseline. This demonstrated

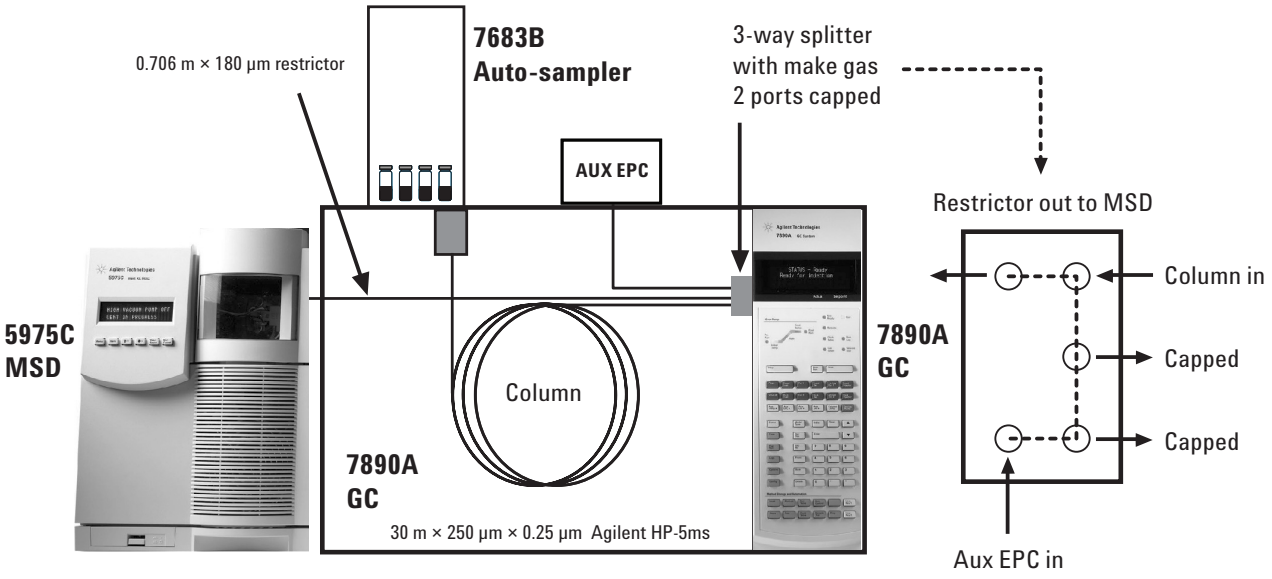

Figure 1. Schematics of GC/MS system configuration and tubing connection for three-way splitter.

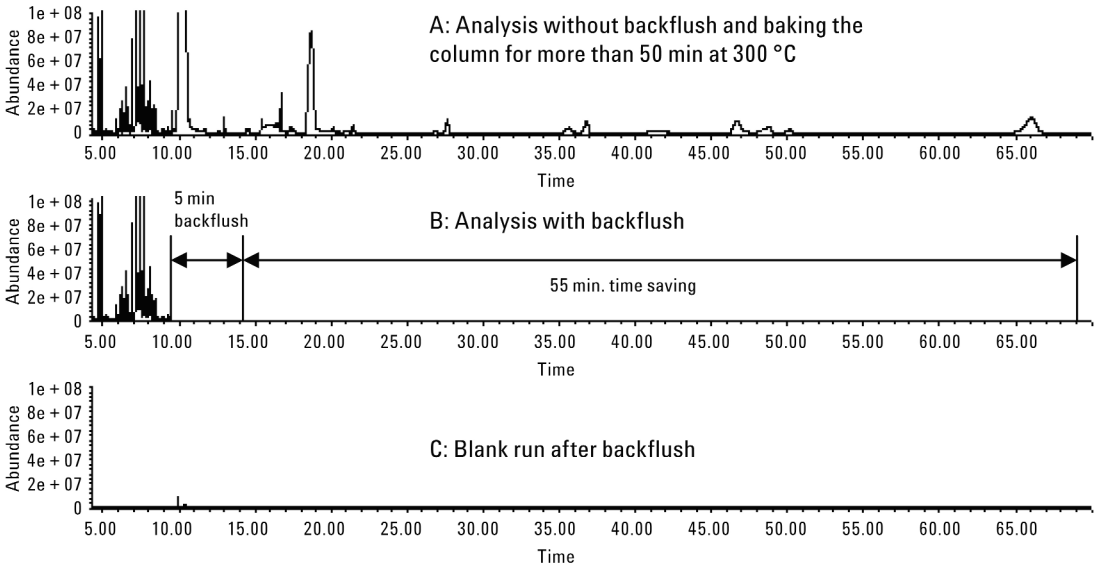

Figure 2. Time savings using backflush.

that backflushing is a perfect solution to avoid both high-temperature baking and retention time shift from run to run. Meanwhile, the faster oven cooling down capability of the Agilent 7890A allows for shorter cycle time. Additional time savings can be realized by using the three-way splitter used in this application, so that the liner and column can be changed without venting the MSD.

Figure 4 shows the total ion chromatograms (TIC) of a spiked milk powder sample. Synchronous SIM/scan was used to monitor ions of interest with high-sensitivity SIM mode and to simultaneously acquire library-searchable scan data in one run. This helped simplify the process of confirming positive or negative results. Figure 5 shows the mass spectra of cyanuric acid tri-TMS derivative (6.335 minutes) and melamine tri-TMS derivative (7.341 minutes), respectively.

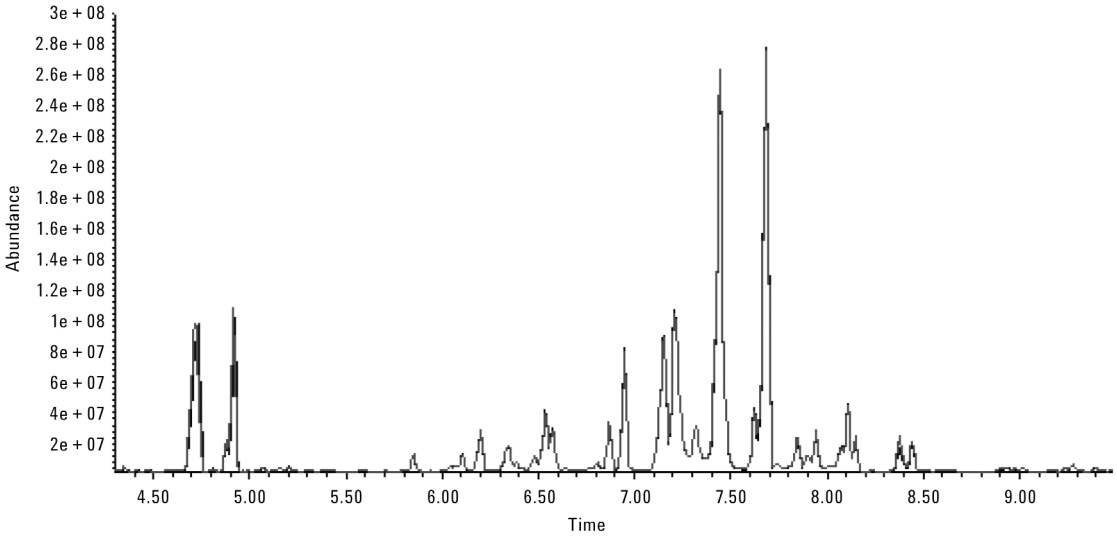

Figure 3. Overlay of total ion chromatogram spectra of two consecutive runs of a powdered infant formula extract.

Linearity and Recovery

A matrix blank milk sample is employed for a linearity experiment. 0.5 g of milk samples were spiked with four levels of cyanuric acid and melamine (10, 20, 80, and 200 µg/g). Excellent linearity was obtained for the two compounds within the range of 10 to 200 µg/g with a correlation coefficient higher than 0.9996.

To check the applicability of the method, a powdered infant formula (blank matrix) spiked with 40 µg/g of targeted analytes was analyzed. Excellent recoveries were obtained, with 96.1% for cyanuric acid and 95.6% for melamine (see Table 3).

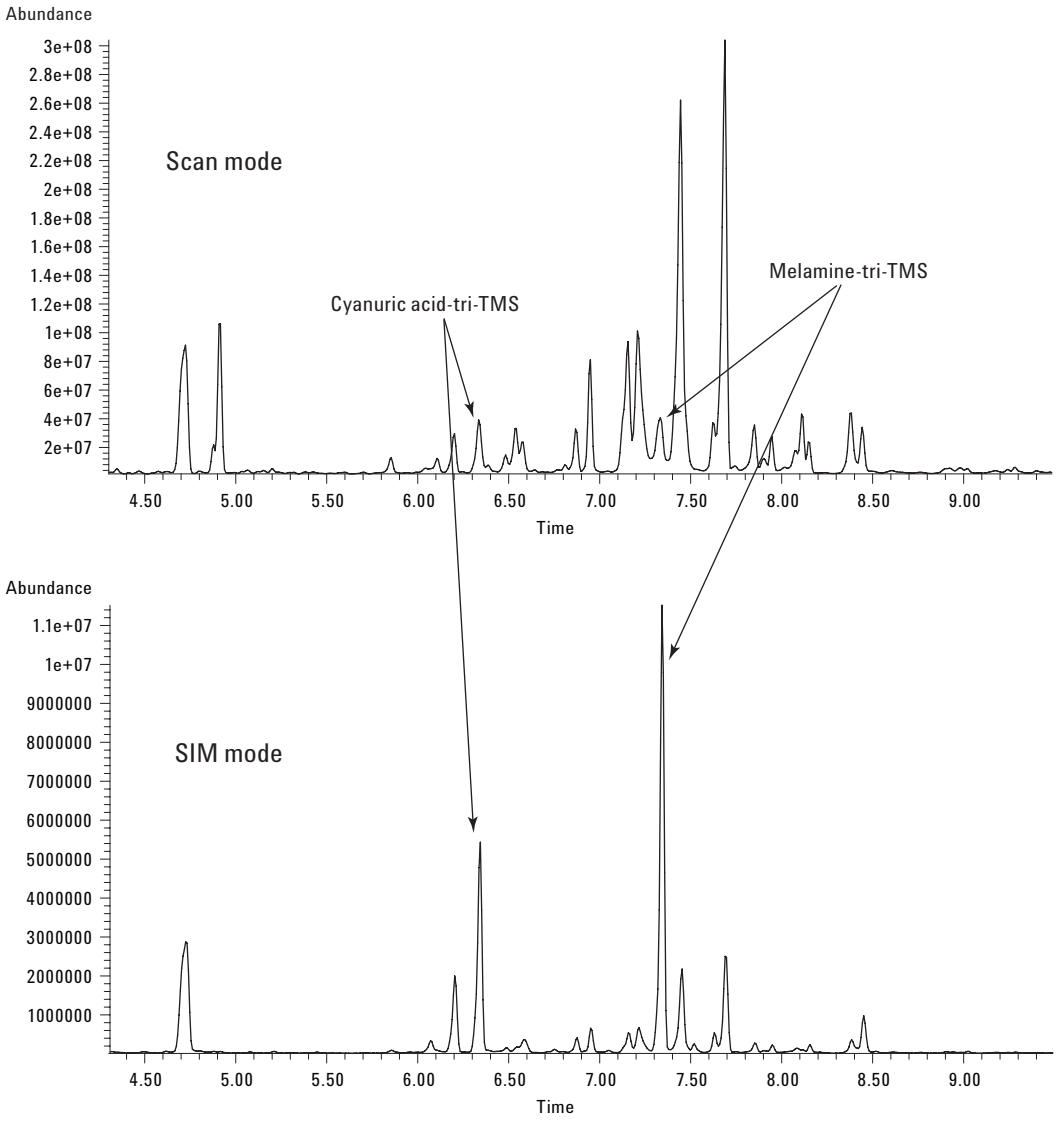

Figure 4. TICs of powdered infant formula sample.

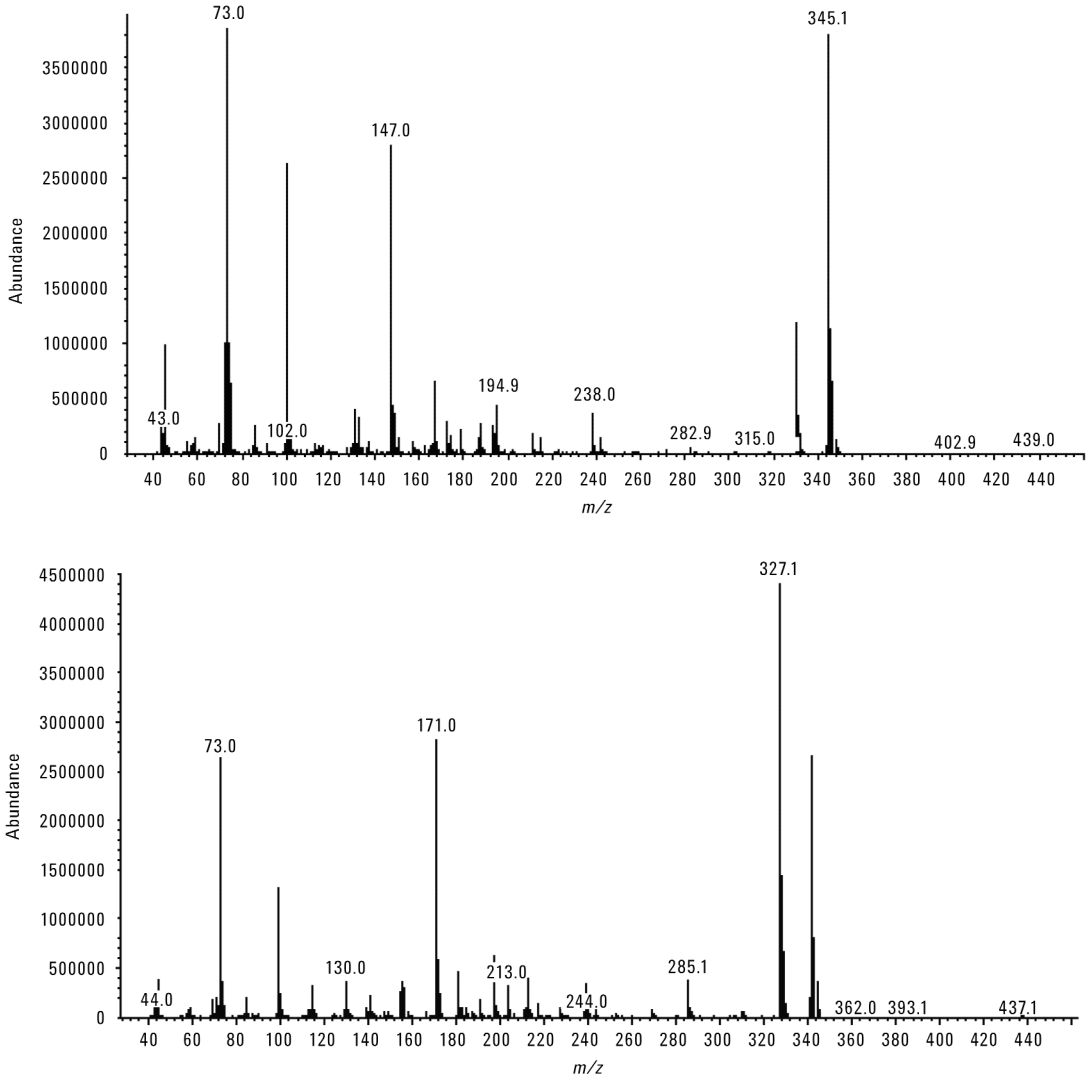

Figure 5. Mass spectra of cyanuric acid tri-TMS derivative (top) and melamine tri-TMS derivative (bottom).

Table 3. Recovery of Spiked Sample

Compound	RT (min)	Spiked level (µg/g)	Measured level (µg/g)	Recovery (%)
Cyanuric acid tri-TMS	6.319	40	38.42	96.1
Melamine tri-TMS	7.333	40	38.23	95.6

Real Sample Analysis

A brand of liquid milk was analyzed with backflush using the previously described method. The two targeted compounds were identified in less than 10 minutes, with cyanuric acid at 34.90 µg/g and melamine at 3.72 µg/g (see Figure 6).

Conclusions

The work described here is a rapid screening and quantitation method for the analysis of melamine and cyanuric acid in milk products that provides excellent linearity and recovery. Using Agilent 7890A/5975C GC/MSD combined with backflushing, the analysis time was cut down to five times shorter than conventional method. This method is fast and suitable for quality control of milk products for the determination of melamine and cyanuric acid.

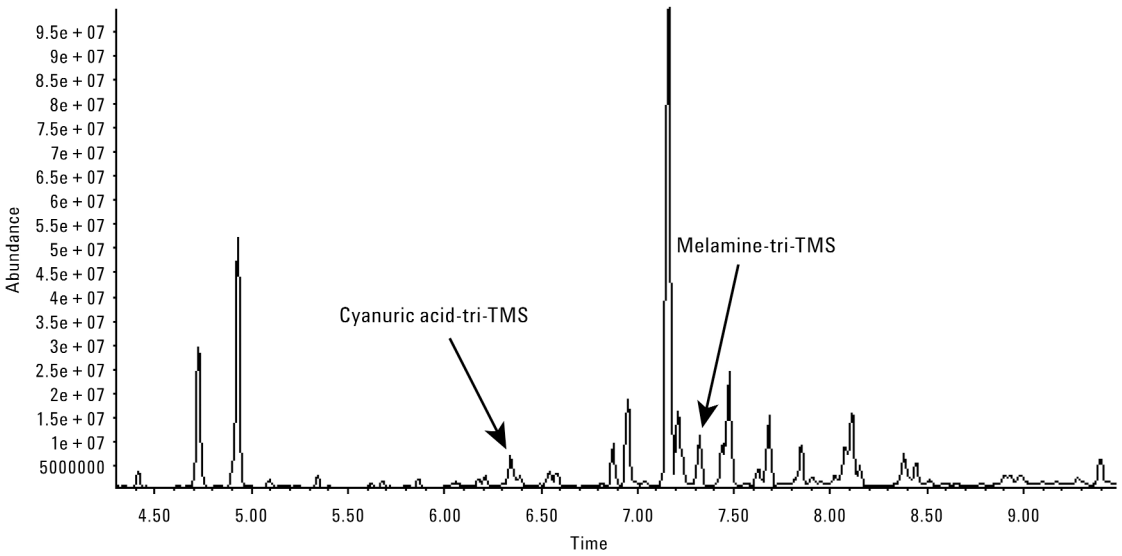

Figure 6. Total ion chromatogram of a contaminated brand of liquid milk.

References

1. U.S. Food and Drug Administration, “GC-MS Method for Screening and Confirmation of Melamine and Related Analogs,” Version 2, May 7, 2007.
2. Mike Szelewski, "New Tools for Rapid Pesticide Analysis in High-Matrix Samples," Agilent Technologies publication 5989-1716EN, Oct.13, 2004.

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Agilent Technologies

Rapid Screening and Confirmation of Melamine Residues in Milk and Its Products by Liquid Chromatography Tandem Mass Spectrometry

Application Note

Food

Authors

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Abstract

This rapid method uses the Agilent 6410 Triple Quadrupole (QQQ) with a cation ion exchange column for the liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis of dairy products for melamine. Milk and milk products are prepared with a simple SPE cleanup method employing the new Agilent SampliQ SCX cartridge. The residue is quantified in the multiple reaction monitoring (MRM) mode. The selectivity of the QQQ can easily eliminate any matrix interferences that may occur in the separation and provide excellent response. The method provides good results with respect to precision, repeatability, and spiked recovery. The recovery of 80 ppb and 50 ppb melamine spikes in milk powder using the external standard calculation is 62.5 and 83.4 percent, respectively, and the RSD is less than 3 percent.

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Introduction

Melamine, a nitrogen-based compound used in industrial and commercial plastics, can cause kidney failure, and has been found in infant formula and other milk products.

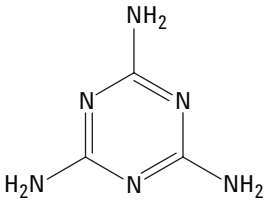

Figure.1 Structure of melamine $C_3H_6N_6$, MW = 126.1199.

In this work, a highly selective, sensitive LC/MS/MS method is developed and, compared to the GC/MS method, requires no derivatization. The method can both confirm and quantify in a single analysis and can achieve very low detection limits in complex matrices such as milk and milk products.

The ZORBAX 300SCX ion-exchange column is simple, fast, and equivalent to the column used in the China GB method and can easily meet the analysis requirements. Therefore, the method can improve lab efficiency/productivity and obtain more reliable and defendable results. It is suitable for the confirmation and quantitation for positive result screening by HPLC.

Experimental

Reagents and Chemicals

The acetonitrile is HPLC grade purchase from Dikma (Beijing, China). The HPLC water is prepared with a Milli-Q system.

Trichloroacetic acid solution: Weigh 50 g trichloroacetic acid and dissolve into 1 L of water.

Ammoniacal methanol solution: Weigh 5 mL ammonium hydroxide and 95 mL methanol

LC Parameters

Column	Agilent ZORBAX 300SCX, 2.1 mm × 150 mm, 5 µm (p/n 883700-704)
Injection volume	10 µL
Flow rate	0.2 mL/min
Temperature	40 °C
Mobile phase	A: 10 mM NH_4 acetate/acetic acid pH adjusted to 3.0 B: ACN A:B = 20:80
Stop time	10 min

MS Parameters

Agilent 6410A LC/MS Triple Quadrupole	
Ion source	Electrospray
Polarity	Positive
Nebulizer gas	Nitrogen
Ion spray voltage	4000 V
Dry gas temperature	350 °C
Dry gas flow rate	9 L/min
Nebulizer pressure	40 psi
Resolution	Q1 (unit) Q3 (unit)

MRM Setting

Rt	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
7 min	Melamine	127	85	200	100	20
		127	68	200	100	35

Sample Preparation

1. Standards solution: dissolve melamine into mobile phase to concentration level at 1, 5, 10, 50, 100, and 500 ppb.
2. Liquid milk, milk powder, yogurt and ice-cream sample preparation:

   2.1 Extraction
   Weigh 2 g of the sample into a 50-mL plastic centrifuge tube with cup, add 15 mL 5% trichloroacetic acid in water solution and 5 mL acetonitrile, sonicate for 10 min, vortex for 10 min, and then centrifuge 10 min at 4000 rpm. Wet filter paper with 5% trichloroacetic acid and filter the supernatant and using a 25.0-mL volumetric flask, bring to
   volume with 5% trichloroacetic acid solution. Transfer 5.0 mL and then add 5 mL water for further cleanup.

   2.2 SPE Cleanup
   Load the above solution onto the SPE cartridge, 6 mL/150 mg SampliQ SCX (p/n 5982-3267). Condition the SPE cartridge before use by washing with 5 mL methanol and then 6 mL water to activate. After loading the sample wash with 5 mL water and then 5 mL methanol, vacuum to almost dry, and elute with 5 mL 5% ammoniacal methanol solution. Control the flow rate at less than 1 mL/min. Dry the eluate under 50 °C nitrogen. Then dissolve the residue (equivalent 0.4 g sample) with 1.0 mL mobile phase, vortex 1 min, and filter via 0.2 µ regenerated cellulose membrane filter (p/n 5064-8222) before injection.

Results and Discussion

Milk and relevant milk products contain hundreds of compounds and it is necessary to distinguish the illicitly added melamine among them. For confirmation and accurate quantification beyond the capability of LC, the LC/MS/MS method is an excellent tool. The high selectivity of the first and third quadrupoles, each working as a "mass filter" in MRM mode, allows selection of the precursor ion and two characteristic fragments, one used for quantitation and the other a qualifier ion (with both present at the corresponding ratio to the standard providing confirmation). Thus, mass spectrometry is a tool that can provide melamine screening laboratories and dairy product manufacturers accurate and precise quantitation and confirmation for samples screened positive.

The chromatograms in Figure 3 show that the Agilent 6410 QQQ determination removes chemical interferences using the high selectivity of the LC/MS/MS in MRM mode. Both the quantitation ion and the qualifier ion have little noise and no matrix interfering peaks, providing accurate and precise quantitation and confirmation. Confirmation is obtained by comparing both the ion ratios and the retention time to the results obtained for melamine standards.

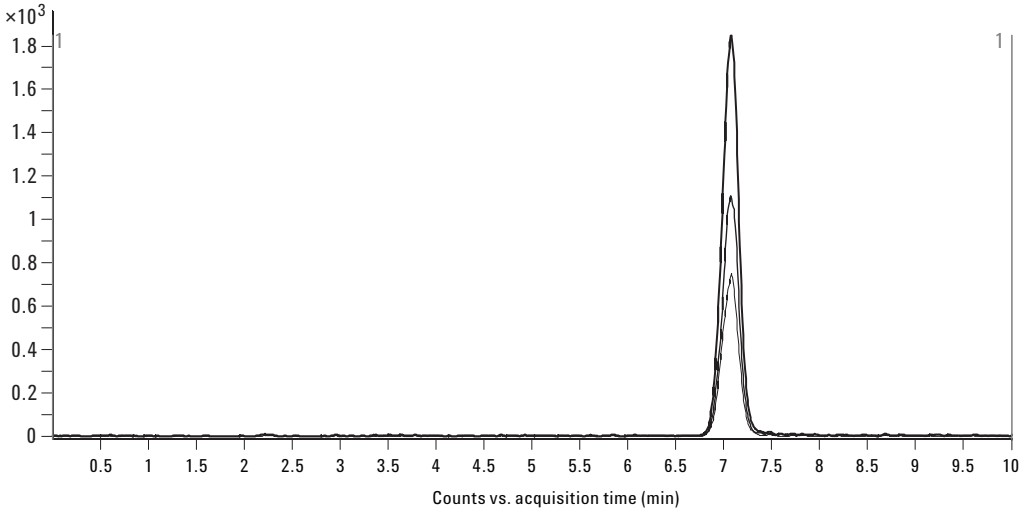

Figure 2. Result in solvent showing the precursor ion and two transition ions at the 10 ppb level.

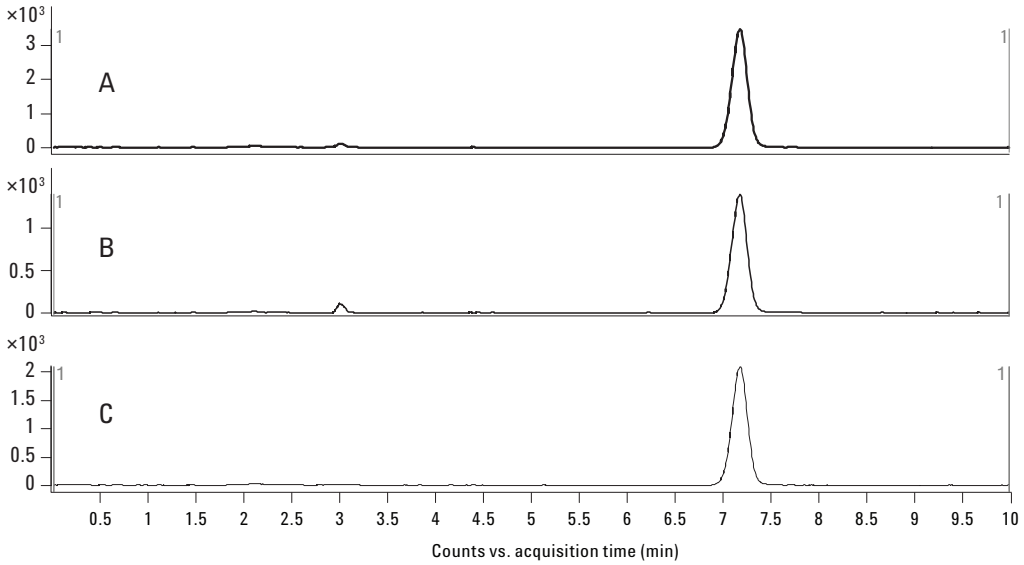

Figure 3. Results of 18.3 ppb level in milk powder with a 10 µL injection, A) total ion chromatogram (TIC) , B) qualifier ion, and C) quantitation ion.

Linearity

Another advantage of QQQ technology is the wide dynamic range for the different levels of sample concentrations as seen in Figure 4.

As shown in Figure 3, it is quite easy to obtain very low detection and, at the same time, analyze high-concentration samples. Samples screened positive by LC/UV detection with possible melamine concentrations above ppm level should be diluted for LC/MS/MS confirmation to avoid contamination of the highly sensitive MS system.

Sensitivity

Using LC/MS/MS excellent sensitivity can be obtained even in complex and dirty matrices. There is almost no background even at very low levels in milk samples.

Figure 5. shows a milk sample spiked at 1 ppb with melamine. Using these data, the calculated result for the limit of quantitation (LOQ) (S/N >10 peak to peak) is 0.5 ppb and limit of detection (LOD) (S/N > 3 peak to peak) is 0.2 ppb.

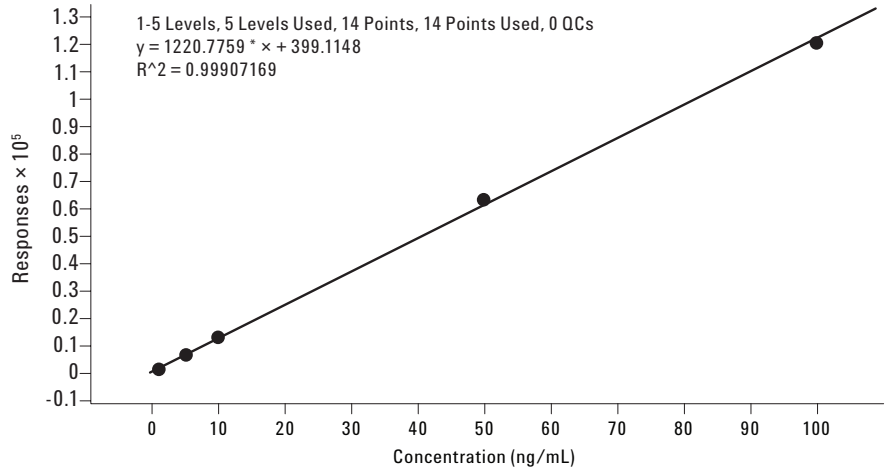

Figure 4. Linearity result for melamine using the MRM quantitation ion.

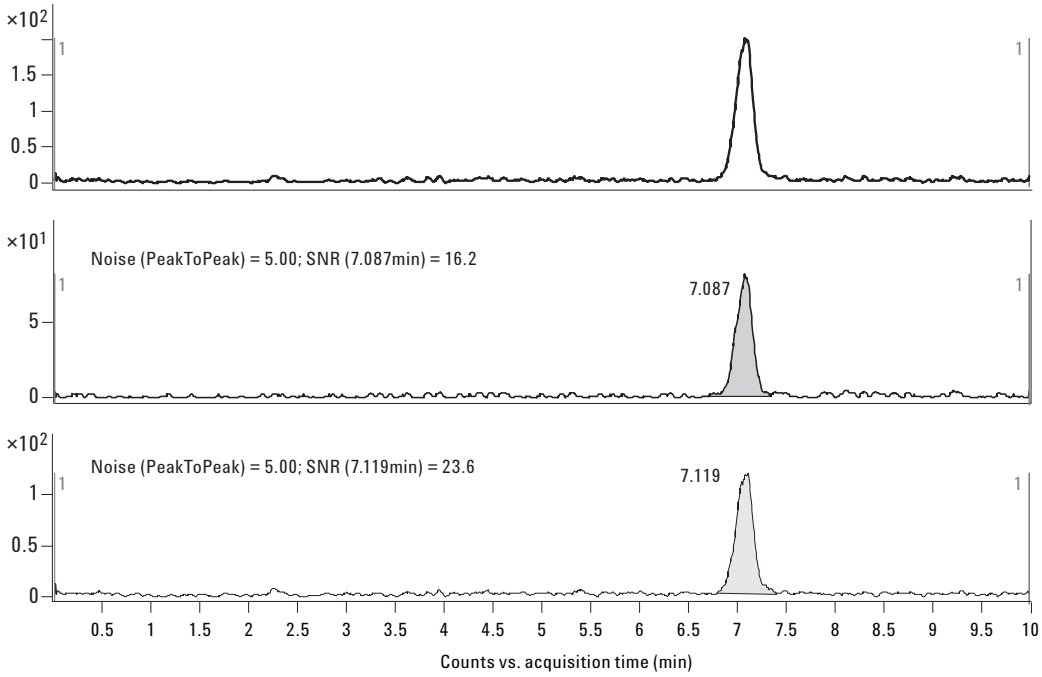

Figure 5. Response of melamine in a milk sample spiked at 1 ppb.

Repeatability

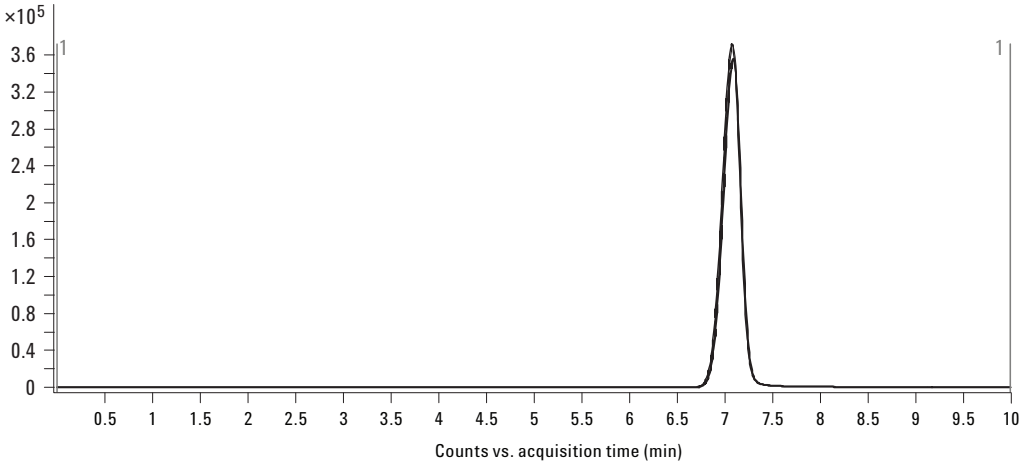

Figure 6. Replicate injections in liquid milk (n = 3) at 10 ppb level.

Table 1. Repeatability in Real Milk Samples with n = 3

Melamine concentration in real milk sample (ppm)	RSD of retention time (%)	RSD of MS response (%)
1	0.30	1.15
100	0.04	1.02

Excellent repeatability of this method is shown in Figure 6 and Table 1. This can ensure good results, even after day-to-day analysis of running samples.

Recovery

Using a calibration curve based on melamine standard in solvent, recovery data in milk powder is shown in Table 2.

Table 2. Recovery in Milk Powder

	Conc. = 80 ppb (n = 3)	Conc. = 50 ppb (n = 3)
Recovery (%)	62.5	83.4
RSD (%)	1.02	2.78

Saturation of the MS detector is observed at about 100 ppb. Using an internal standard method is recommended for future analysis with stable isotope labeled melamine.

Conclusions

A sensitive and specific method for the detection and quantitation of melamine in milk and milk products has been demonstrated. The method is robust and allows for the analysis of a large number of samples in complex matrices. Derivatization is not needed, and the method provides confirmation and quantitation in a single analysis at very low detection limits. This method can be readily used for confirmation of positive results obtained with less selective LC screening methods. The results of this study show that the Agilent 6410 LC/MS Triple Quadrupole, SampliQ SCX SPE cartridges, and a ZORBAX 300SCX HPLC column provide a robust, sensitive, and repeatable methodology.

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Agilent Technologies

Trace-Level Analysis of Melamine in Milk Products on Agilent 7890A/5975C GC/MSD Using a New Agilent J&W DB-5ms Ultra Inert Column and SampliQ SCX Cartridges

Application Note

Gas Chromatography/Mass Spectrometry/Food Safety

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Abstract

A GC/MS method is presented for the quantitative determination and confirmation of melamine residues in milk products. The milk sample was cleaned up using Agilent's new SampliQ SCX SPE cartridges before derivatization. The derived extracts were analyzed by GC/MS with EI in synchronous SIM/scan mode on a new Agilent J&W DB-5ms Ultra Inert column. The method has good linearity in the range of 0.025 to 2.000 µg/mL with a correlation coefficient of 0.9986. The average recoveries of melamine, for milk spiked at 0.080, 0.800, and 1.600 mg/kg, were over 80 percent, with RSDs of less than 5 percent. The LOD is 0.0025 mg/kg in the milk matrix, which is suitable for trace-level analysis of melamine in milk products.

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Introduction

Melamine is an organic base chemical most commonly found in the form of white crystals rich in nitrogen. Recently, melamine in adulterated milk product has attracted much attention all over the world. The main analytical methods available for melamine quantification in milk involve GC/MS, HPLC, and LC/MS, such as China GB/T method 22388-2008 [1]. An HPLC method can be used to quantitatively analyze melamine at ppm level. But it is inadequate for qualitative analysis and trace-level analysis. LC/MS is a sensitive technique for quantitative and qualitative analysis. However, due to the high price of equipment, it is not widely used in practical applications. GC/MS is an economic and commonly used technique for most of analytical laboratories.

Milk products are complex matrixes that contain interfering compounds, such as protein and fat. These compounds may interfere with the analysis of the target analytes or contaminate the analytical system if the sample is introduced without selective sample preparation.

Solid-phase extraction (SPE) is commonly used as a sample preparation method for a variety of analytical procedures. Agilent's mixed-mode SampliQ SCX polymeric resin is a sulfonic acid-modified divinyl benzene polymer with both ion exchange and reverse-phase retention properties. The SampliQ SCX resin exhibits excellent retention for both basic and neutral compounds. It ensures fast, reliable extraction of basic compounds like melamine.

GB/T method 22388-2008 requires melamine analysis with an LOQ of 0.05 mg/kg in a GC/MS method. For a complex matrix like milk sample, an inert GC/MS column is crucial for trace-level analysis. The new Agilent J&W Ultra Inert column allows for the best and most consistent column inertness [3], providing better sensitivity and peak shape for active compounds and reliable results for trace-level analysis. Combined SampliQ SCX SPE with the Agilent J&W DB-5ms Ultra Inert column, a sensitive GC/MS method, is put forward on trace-level analysis of melamine in milk products.

Experimental

Standards and Reagents

The standards and reagents used in the experiment are listed in Table 1.

A stock solution 1,000 µg/mL of melamine was prepared in methanol. Working solutions were prepared by diluting the stock solution with methanol. After being transferred to an autosampler vial, the 1-mL working solution was allowed to evaporate to dryness with nitrogen. 600 µL pyridine and 200 µL BSTFA were then added, vortexed briefly to mix, and incubated at 70 °C for 30 minutes. With this procedure, a series of standard solutions of melamine trimethylsilyl (TMS) derivatives (0.025, 0.050, 0.200, 0.500, 1.000, and 2.000 µg/mL) were prepared for linearity analysis.

Table 1. Standards and Reagents

Melamine	> 99% purity
Methanol	HPLC grade
Pyridine	Certified A.C.S. reagent
1% trichloroacetic acid (TAA) solution	Dissolve 10.0 g of TAA in reagent water. Dilute to 1.0 L with reagent water.
22 g/L lead acetate solution	Dissolve 22.0 g of lead acetate in reagent water. Dilute to 1.0 L with reagent water.
5% ammonium hydroxide solution	Mix 5 mL of ammonium hydroxide and 95 mL of methanol together for use.
Silylating reagent	BSTFA with 1% TMCS: bis(trimethylsilyl) trifluoroacetamide with 1% Trimethylchlorosilane

Instruments

Table 2 lists the GC chromatography and mass spectrometry conditions of the method.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Instruments	Agilent 7890A/5975C GC/MSD Agilent 7683 Automatic Liquid Sampler (ALS)
Column	Agilent J&W DB-5ms Ultra Inert, 30 m × 0.25 mm × 0.25 µm (P/N 122-5532UI)
Inlet temperature	EPC, split/splitless @ 250 °C
Injection volume	1 µL, split 3:1
Carrier gas	Helium, constant flow mode, 1.3 mL/min
Oven program	75 °C (1 min); 30 °C/min to 300 °C (2 min)
Transfer line	290 °C
MS Conditions	
MS	EI, SIM/scan
Solvent delay	4.2 min
MS temperature	230 °C (source); 150 °C (quad)
Scan mode	Mass range (40 to 450 amu)
SIM mode	Ion (342, 327*, 171, 99)

*Quantitative ion

Sample Preparation

Sample preparation was performed according to the scheme in Figure 1. A SampliQ SCX SPE cartridge (60 mg, 3 mL, P/N 5982-3236) was used to clean up sample extracts. All SPE elution steps, including conditioning, sample loading, washing, and elution were performed without the application of vaccum. Vacuum was only applied to dry the cartridges.

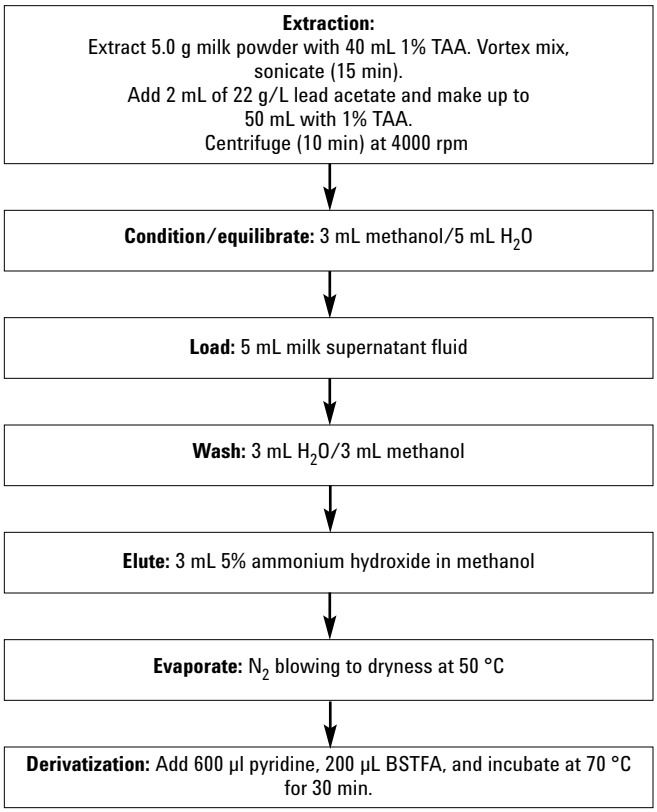

Figure 1. Scheme of sample preparation process.

The derivatization of melamine is shown is Figure 2.

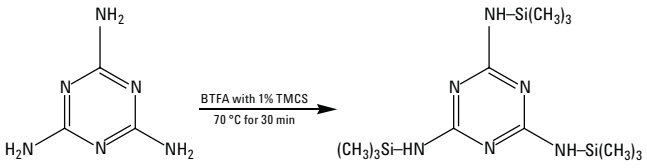

Figure 2. Melamine derivatization reaction.

Results and Discussion

GC/MS Analysis of Melamine

GC/MS was used for the identification (full-scan mode) and quantification (SIM mode) of melamine. It helped simplify the process of confirming positive or negative results.

Figure 3A shows the chromatogram of a sample that was only extracted with methanol without any SPE cleanup [4]. It highlights that there was much sample matrix interference in milk extract for the analytes of interest. Especially after 10 minutes, large amounts of high boilers eluted from the column. Undoubtedly, it increased the analysis time to ensure that the late eluters were eluted. Furthermore, it could contaminate the analytical system and shorten the lifetime of the ion source.

Figure 3B shows the chromatogram of a sample prepared according to the procedure in Figure 1. Figure 3C is the chromatogram of melamine tri-TMS for reference. The SCX SPE effectively removed interferences from the sample matrix. Downtime is reduced due to less detector contamination.

Figure 4 is the corresponding mass spectrum of melamine tri-TMS in Figure 3. After SPE cleanup, it eliminates some interference ions from the matrix and the mass spectra shows better "match" factors over 90. It further demonstrates that it makes the qualitative and quantitative analysis easier and more accurate.

As you can see in Figure 3, sharp, symmetrical peaks were observed for melamine tri-TMS on Agilent J&W DB-5ms Ultra Inert GC columns. Sharper peaks greatly enhance the height of the signal and therefore provide better signal-to-noise ratios and greater sensitivity. The ultra inert column ensures minimal compound adsorption, for more accurate quantification. It proved that Agilent J&W Ultra Inert GC columns allow you to perform trace-level analysis of melamine with the utmost confidence.

Linearity and Recovery

The linearity of the method was studied using the standard working solutions after derivatization. Figure 5 is the calibration curve for melamine tri-TMS. The method has good linearity in the range of 0.025 to 2.000 µg/mL, with a correlation coefficient of 0.9986.

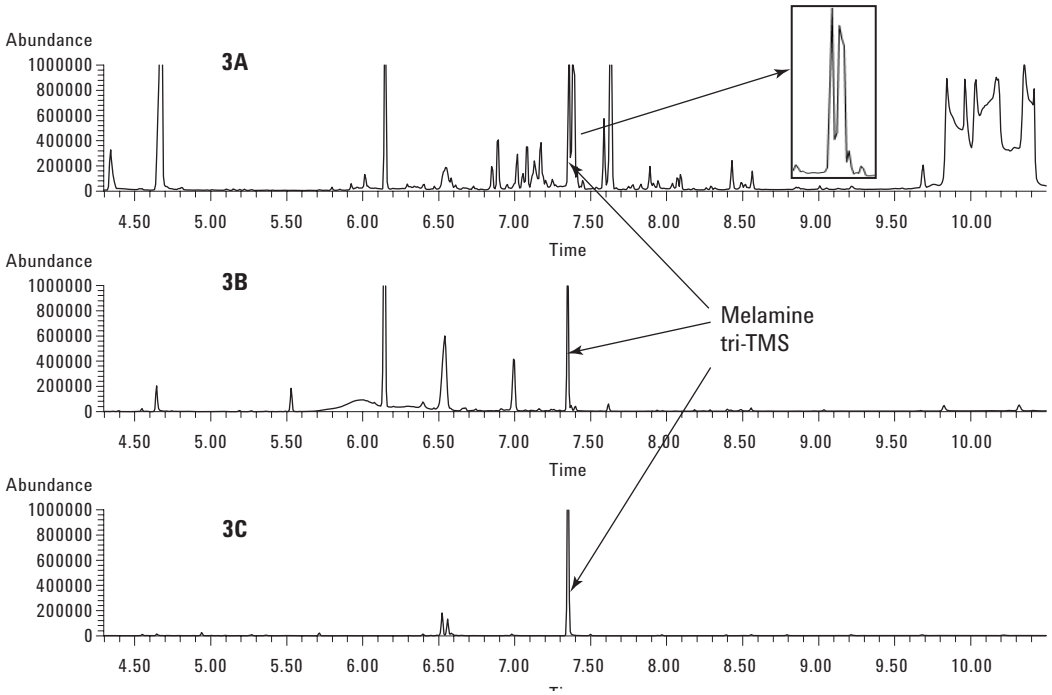

Figure 3. GC/MS SIM chromatogram of melamine tri-TMS. (3A: Sample without SPE cleanup; 3B: Sample with SPE cleanup; 3C: Standard)

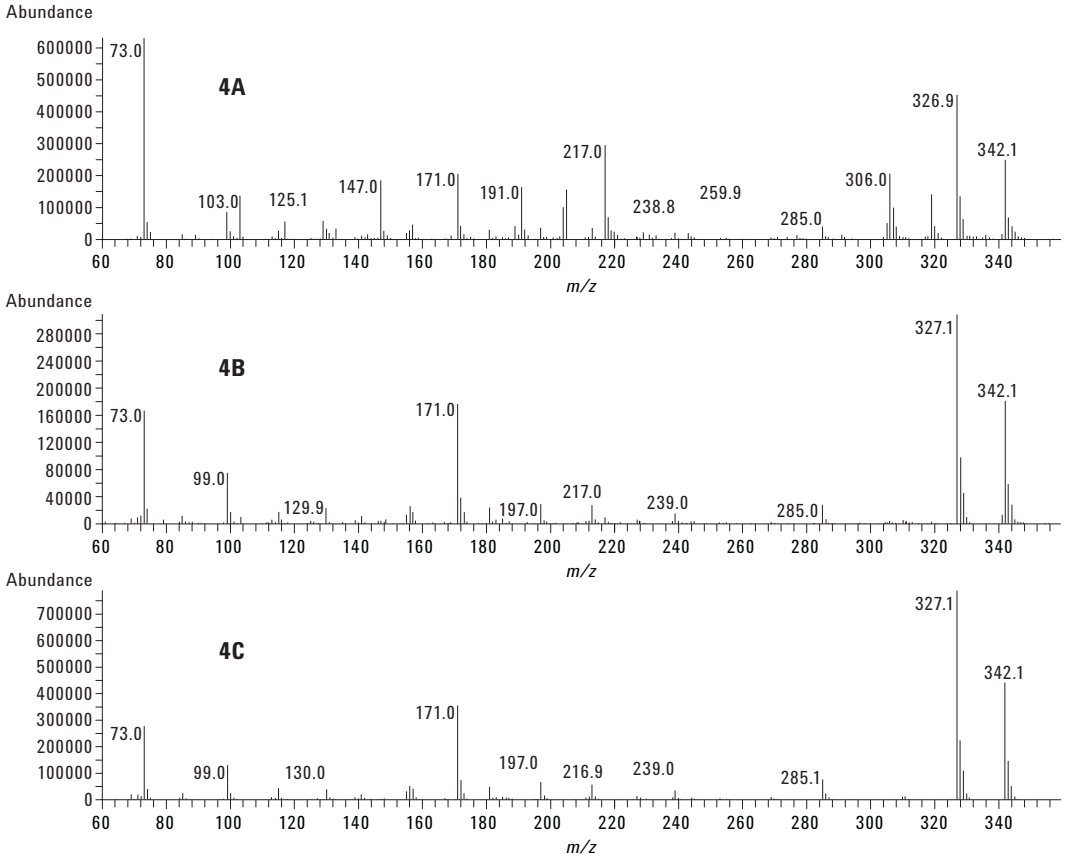

Figure 4. GC/MS mass spectrum of melamine tri-TMS. (4A: Sample without SPE cleanup; 4B: Sample with SPE cleanup; 4C: Standard)

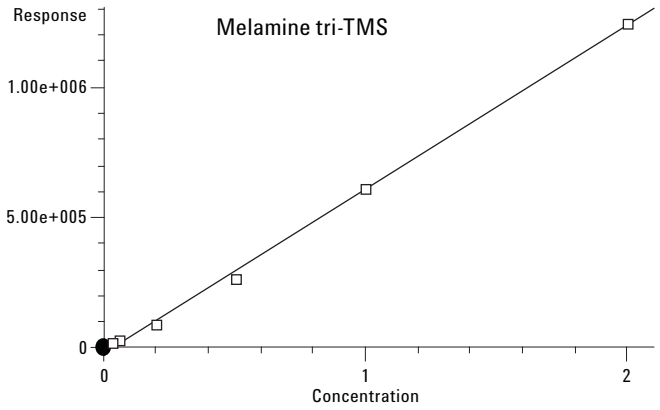

Figure 5. Calibration curve for melamine tri-TMS.

The recovery of the method was evaluated for melamine spiked at 0.080, 0.800, and 1.600 mg/kg levels. For each concentration level, the repeatability was evaluated on six duplicate samples. The spiked samples were treated according to the sample-preparation procedure described above. The recovery and repeatability data are listed in Table 3. The method resulted in good recoveries (over 80 percent) and repeatability with RSDs less than 5 percent at three levels. The LOD is calculated from the level of 0.08 mg/kg with a signal-to-noise ratio of 3. The LOD is 0.0025 mg/kg in the milk matrix, which is applicable for the trace-level analysis of melamine.

Table 3. Recovery and Repeatability of Spiked Samples

Compound	Spiked level (mg/g)	Recovery (%)	RSD (%) (n = 6)
Melamine tri-TMS	0.080	82.1	2.04
	0.800	82.8	4.88
	1.600	80.8	3.58

Conclusions

This application demonstrates a sensitive GC/MS method for melamine analysis in milk products using an Agilent J&W DB-5ms Ultra Inert GC column and SampliQ SCX SPE cartridges. It is suitable for trace-level analysis of melamine in raw milk and diary products.

References

1. China GB/T 22388-2008 Determination of Melamine in Raw Milk and Diary Products.
2. U.S. Food and Drug Administration, GC-MS Screen for the Presence of Melamine and Cyanuric Acid. Revised April 25, 2007.
3. "Agilent J&W Ultra Inert GC Columns: A New Tool to Battle Challenging Active Analytes," Agilent Technologies publication 5989-8685EN, May 29, 2008
4. "Rapid Screening of Melamine and Cyanuric Acid in Milk Products Using Agilent J&W HP-5ms GC column and Agilent 7890A/5975C GC/MSD with Column Backflushing," Agilent Technologies publication 5989-9866EN, October, 2008

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Agilent Technologies

Rapid Screening and Confirmation of Melamine and its Analogs in Baby Formula and Soy Products Using Triple Quadrupole GC/MS and Backflushing

Application Note

Food

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Abstract

A rapid method for the screening and confirmation of melamine, ammelide, ammeline and cyanuric acid in baby formula and soy meal was developed using the Agilent 7890A/7000A Series Triple Quadrupole GC/MS and backflushing with a Purged Ultimate Union. The extraction and derivatization procedures are the same as those used in the FDA GC/MS method. Excellent linearity ($R^2 > 0.99$) was obtained in the range of 0.16 to 2.5 ppm, with run times less than 15 minutes.

Agilent Technologies

Introduction

The adulteration of food with melamine has quickly become an international problem as it has been detected in baby formula produced in the US, chocolates distributed in Canada, biscuits sold in the Netherlands, condensed milk in Thailand and eggs in Hong Kong. In response, many countries have established allowable limits for melamine, with the FDA maximum residue limit (MRL) as 1 part per million (ppm) for infant baby formula and 2.5 ppm for other products. The FDA GC-MS screening method [1] is capable of detecting melamine and its analogs (ammeline, ammelide and cyanuric acid) at 2.5 ppm. However, the FDA import alert of February 2009 requires that a testing method with a sensitivity of 0.25 ppm for melamine and its analogs be used to assure compliance to the MRLs. Therefore this method cannot be used to screen for melamine and its analogs under the new regulations, and confirmation would require an additional orthogonal method.

This application note describes a modification of the FDA GC-MS method for use on the new Agilent 7000A Series Triple Quadrupole GC/MS. The new method, which does not require a change in sample extraction and derivatization procedures, employs a purged union GC column configuration and backflushing to provide run times under 15 minutes. Melamine and its analogs can all be detected at 0.25 ppm, with highly reproducible and accurate quantification. Most importantly, this method provides screening, quantification and confirmation of melamine and its analogs, all in one short run.

Experimental

Standards and Reagents

The standards and reagents used are listed in Table 1. Stock solutions of melamine, ammelide, ammeline and cyanuric acid, each at a concentration of 1,000 µg/mL, were separately prepared in a mixture of DEA/H20 (20/80) and stored at 4 °C. Internal standard (2,6-Diamino-4-chloropyrimidine, or DACP) was prepared at a concentration of 57.7 ng/mL in pyridine. The above solutions were used to prepare matrix-matched standards as described in the FDA method [1]. Matrix samples were generously provided by the FDA.

Table 1. Standards and Reagents

Standard	Melamine	Sigma-Aldrich	>99% purity
	Cyanuric acid	TCI-America	>98.0%
	Ammelide	TCI-America	>98.0%
	Ammeline	TCI-America	>95.0%
	Internal standard†	Sigma-Aldrich	98%
Solvent	Diethylamine (DEA)	Sigma-Aldrich	SigmaUltra grade
	Pyridine	Fisher Scientific	Certified A.C.S. reagent
	Acetonitrile	Fisher	HPLC grade
Silylating reagent	BSTFA with 1% TMCS* (SYLON BFT)	Sigma-Aldrich	Derivatization grade

† DACP (2,6-Diamino-4-chloropyrimidine)
* BSTFA: bis(trimethylsilyl)trifluoroacetamide, TMCS: Trimethylchlorosilane

Instruments

The experiment was performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet, an Agilent 7000A Series Triple Quadrupole GC/MS with Triple-Axis Detector, and an Agilent 7683B automatic liquid sampler (ALS). The split/splitless inlet was fitted with a long-lifetime septum (p/n 5183-4761) and a deactivated, splitless single taper injection liner (p/n 5181-3316). Injections were made using a 10-µL syringe (p/n 9301-0714). The instrument conditions are listed in Table 2.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Column	Two 15 m × 0.25 mm × 0.25 µm HP-5ms columns (p/n 19091S-431)
Inlet temperature	280 °C
Inlet pressure	12.9 psi
Carrier gas	Helium, constant flow mode, 1.2 mL/min
Pulsed splitless	25 psi at 0.5 min
Oven program	100 °C (1 min hold), 10 °C/min to 210 °C
Column velocity	41 cm/s
Injection volume	1 µL
Transfer line temperature	290 °C
GC Post-Run Conditions	
Backflush device	Purged Ultimate Union (p/n G3186-60580) controlled by a Pressure Control Module (p/n G3476-60501)
Backflush conditions	–3.6 mL/min at 300 °C for 1.3 min
MS Conditions	
Tune	Autotune
Delta EMV	400 V
Acquisition parameters	EI; selected reaction monitoring
Solvent delay	6 minutes
MS temperatures	Source 230 °C; Quadrupoles 150 °C

Sample Preparation

A 0.5-g amount of a representative portion of the sample was weighed into a 50-mL polypropylene centrifuge tube. An extraction solvent of DEA/Water/Acetonitrile (10/40/50) was prepared, and 20 mL added to the weighed sample. Diethylamine dissociates the melamine-cyanuric acid complex, thus reducing the risk of false negative measurements. DEA also improves the solubility of ammelide and ammeline, which have extremely low solubility in traditional extraction solvents. The sample was capped, vortex mixed, and then sonicated for 30 minutes. After the sample was centrifuged at 5,000 g or higher for 10 minutes, the supernatant fluid was filtered through a 0.45-µm nylon filter.

Derivatization

A 160-µL amount of the filtrate was transferred to a glass GC vial. The extract was evaporated to dryness under a stream of nitrogen at approximately 70 °C, and 600 µL of ISTD and 200 µL of BSTFA with 1% TMCS were added. The sample was vortex mixed and incubated at 70 °C for 45 minutes before injecting.

Analysis Parameters

The parameters used in the analysis of melamine and its analogs, as well as the internal standard, are shown in Table 3.

Table 3. Analysis Parameters

		Triple Quadrupole GC/MS		
Compound	**RT**	**SRM**	**Dwell time (ms)**	**Collision energy (EV)**
Melamine	12.467	327 → 171	20	17
		342 → 285	150	20
		342 → 213	150	22
Ammelide	10.801	344 → 171	50	22
		344 → 214	50	15
		329 → 171	50	20
Ammeline	11.748	328 → 171	50	25
		343 → 214	50	20
		343 → 171	50	30
Cyanuric acid	9.613	345 → 215	50	8
		345 → 188	50	12
		330 → 215	50	4
DACP (ISTD)	11.185	273 → 237	150	12
2,6-Diamino-4-chloropyrimidine		273 → 99	150	20

Results and Discussion

Backflushing with a Purged Ultimate Union System

A backflushing configuration was employed to remove higher boiling substances from the column prior to each subsequent run by flushing late eluting peaks out of the inlet split flow vent instead of driving them though the entire column and into the MSD. Backflushing reduces chemical noise and the cycle time of the analysis, thus increasing throughput. System uptime is also increased, due to reduced maintenance of the columns and MS detector. The suite of Agilent Capillary Flow Technology modules comprises a proprietary solution that enables easy and rapid backflushing with small dead volumes for improved resolution, and ferrules and fittings that eliminate leaks. All Capillary Flow Technology modules require the use of an Auxiliary Electronic Pneumatic Control (EPC) module or a Pneumatic Control Module (PCM) to provide a precisely-controlled second source of gas that directs the column flow to the appropriate column or detector. In normal operation, the PCM pressure is at or slightly above the pressure of the carrier gas through the column. During backflush, the inlet pressure is dropped to 1 psi and the PCM pressure is increased, forcing the flow to reverse through the column and out the purged inlet.

A unique, alternative approach to backflushing is the use of a Capillary Flow Technology device in the middle of the analytical column [2, 3]. Instead of using a 30-m column, two 15-m columns are used and connected by an ultra-low dead volume Purged Ultimate Union (Figure 1). The PCM adds just enough makeup gas to match that from the first column. Therefore, there is very little flow addition and subsequent decrease in sensitivity due to sub-optimal carrier gas flows into the mass spectrometer. Backflushing in this configuration is accomplished by reducing the flow and pressure in the first column and increasing them in the second column.

Figure 2 shows an example of backflushing with the purged union configuration. The top chromatogram shows six standards, where the third peak is considered the last analyte of interest and the fourth peak is the first of the late-eluting interferences. The middle chromatogram shows (a) the same

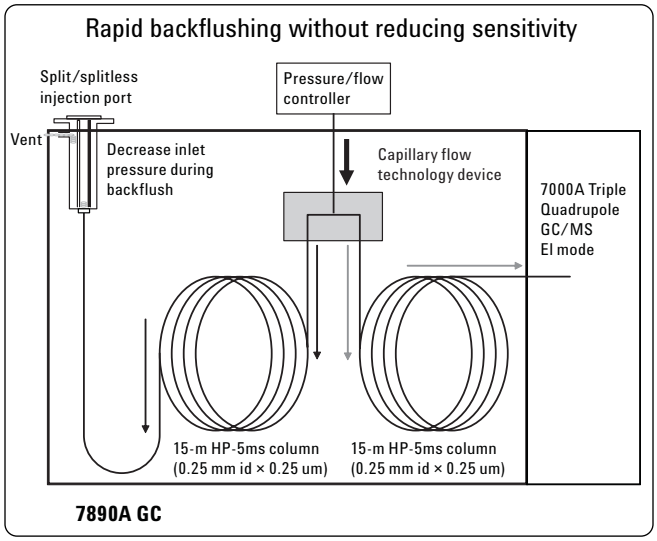

Figure 1. Schematic of the Purged Ultimate Union GC/MS configuration.

standard with backflushing beginning at 10.1 minutes, where flow is dropped in the first 15-m column and (b) where the flow in the second column is increased. The time between points a and b is the residence time of the last analyte compound in the second column. The last analyte is retained, but the late eluters never enter the MS detector. The bottom chromatogram demonstrates the lack of carryover in a subsequent blank run. Alternatively, backflushing can begin after the last peak of interest has eluted (point b). This eliminates the need to experimentally determine the residence time of the last target compound in the second column, while slightly increasing the cycle time.

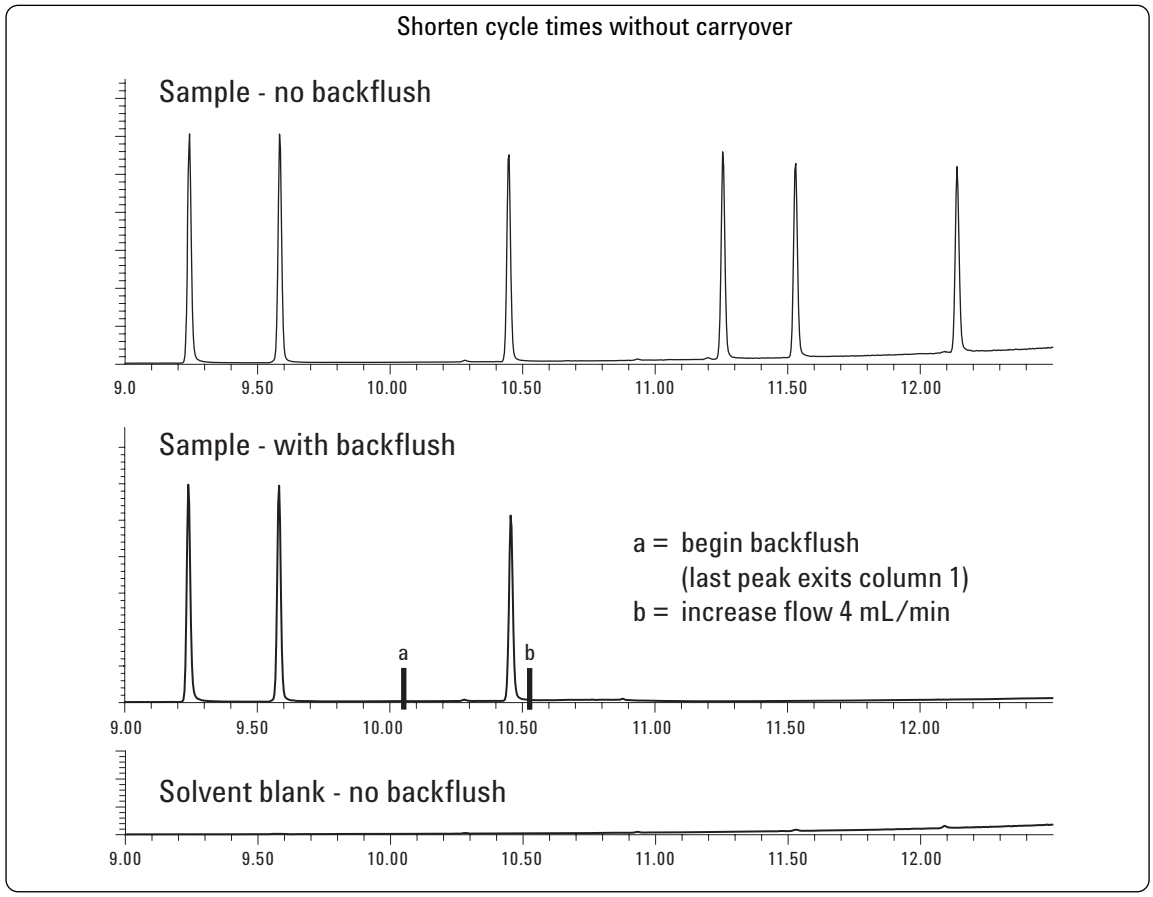

Figure 2. Backflushing with a purged ultimate union configuration. Top: no backflushing. Middle: Backflushing beginning at 10.1 minutes (a) until the third analyte elutes off the second column (b). Bottom: Subsequent blank injection showing no carryover.

Analysis of Melamine and its Analogs

The method developed on the Triple Quadrupole GC/MS system provides excellent separation and analysis of melamine, ammelide, ammeline and cyanuric acid in one run, and in less than 15 minutes (Figure 3). The significant improvement in the sensitivity and selectivity of the new Triple Quadrupole GC/MS method versus the GC/MS SIM method is vividly illustrated in Figure 4. While the new method provides a very clean analysis of the quantifying transition of melamine at 0.25 ppm, the GC/MS SIM method is less effective at reducing chemical noise at 2.5 ppm, using any of the SIM ions.

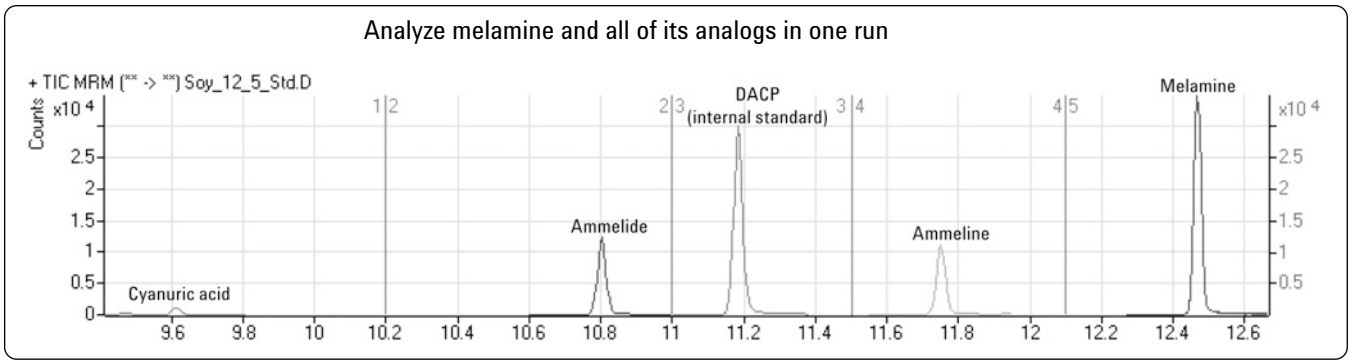

Figure 3. Reconstructed Total Ion Current Chromatogram (RTICC) resulting from SRM analysis, illustrating the resolution of melamine and its analogs.

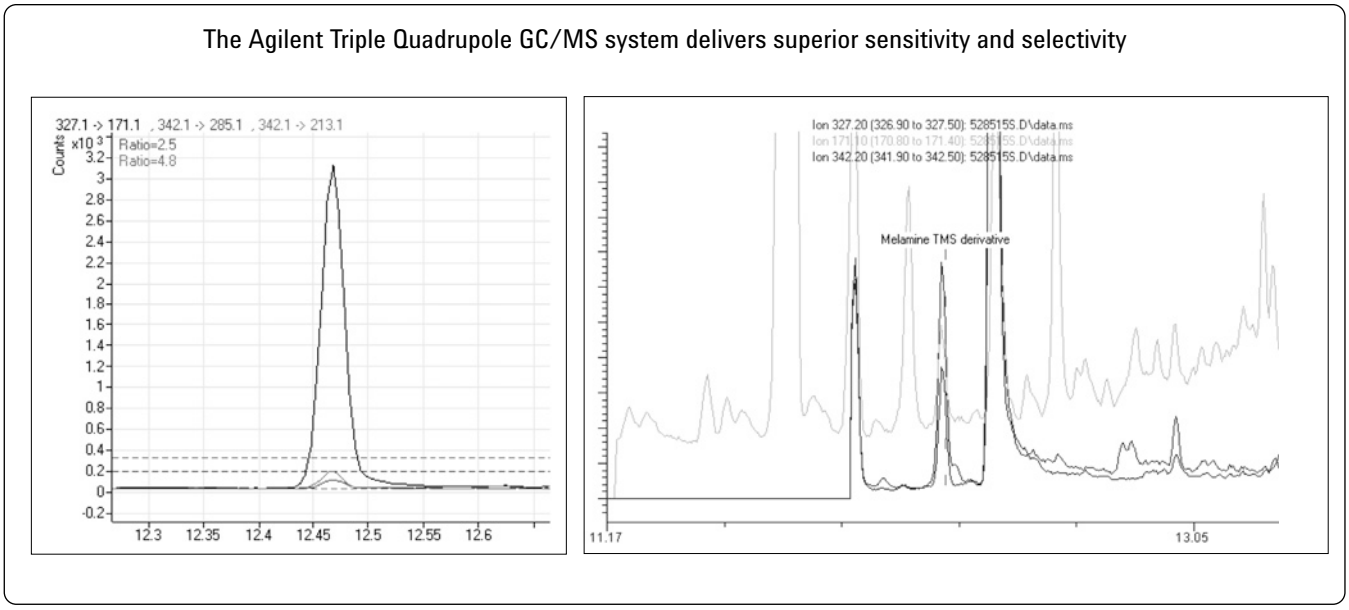

Figure 4. Comparison of detection of 0.25 ppm melamine in soy meal using the Triple Quadrupole GC/MS method (a), versus the GC/MS SIM method at 2.5 ppm (b). The quantifying transition used with the Triple Quadrupole GC/MS method was m/z 327.1→171.1, and the qualifying transitions were m/z 342.1→295.1 (2.5% of the peak area of the quantifying transition) and m/z 342.1→217.1 (4.8% peak area). The uncertainty bands are shown in (a) as well. The SIM ions used in the GC/MS method were m/z 342.2, 327.2, and 171.1 (b).

Sensitivity and Quantification

Each of the standards for melamine and its three analogs was added to matrix (both baby formula and soy meal) at concentrations of 0.78, 1.25, 3.9 and 12.5 ng/mL, corresponding to detection levels of 0.16 to 2.5 ppm. Calibration curves were constructed for each of the four compounds in each matrix.

Figures 5 and 6 illustrate the excellent linearity obtained for melamine and its three analogs, with R^2 values very close to 1.00. The accuracy of quantification was also very good for all four compounds in both matrices as illustrated in Tables 4 and 5.

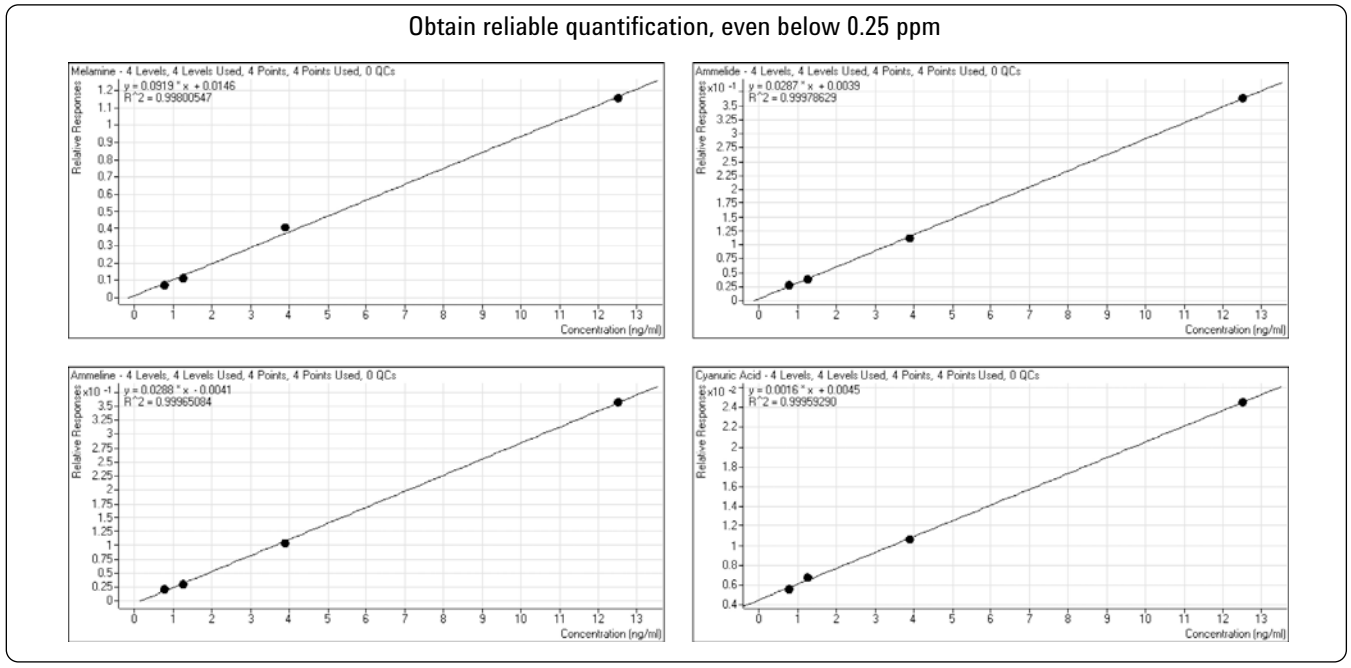

Figure 5. Calibration curves for quantification of melamine and its derivatives in baby formula based on a linear fit.

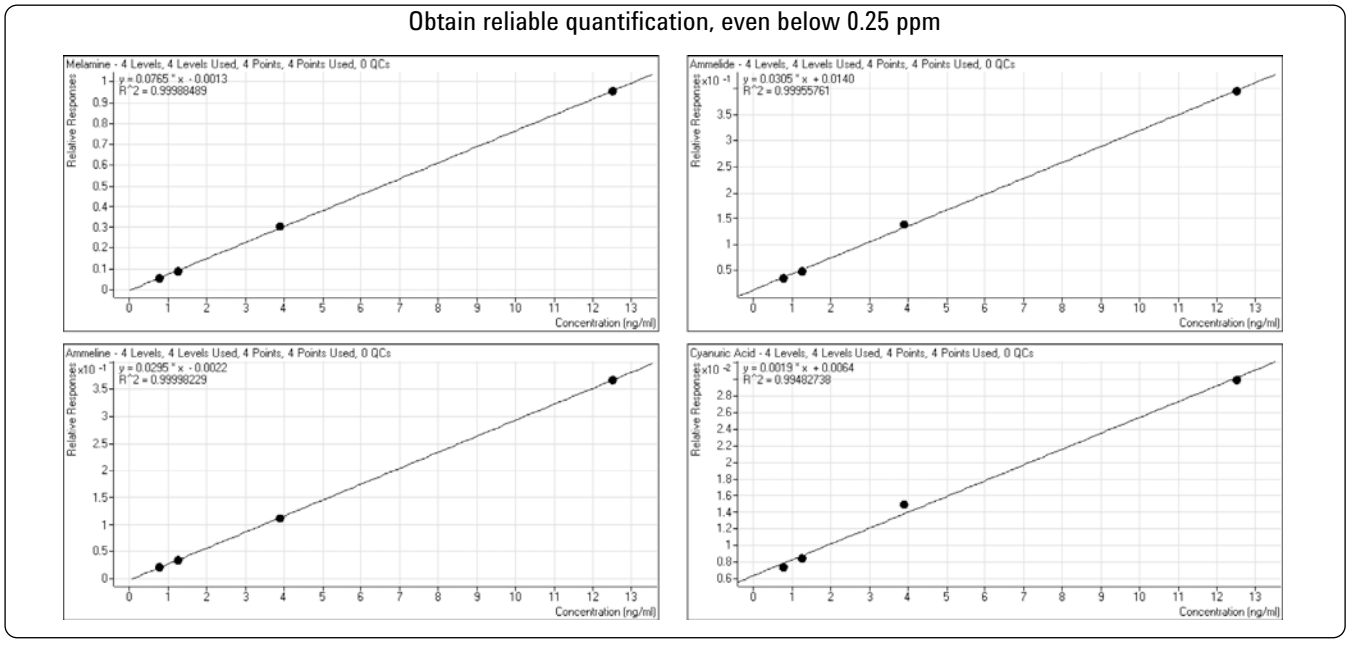

Figure 6. Calibration curves for quantification of melamine and its derivatives in soy meal based on a linear fit.

Table 4. Calibration Data for Quantification of Melamine and its Derivatives in Baby Formula Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.79	101.3
	1.25	1.23	99.1
	3.90	4.39	112.5
	12.5	12.50	100.0
Ammelide	0.78	0.86	110.3
	1.25	1.25	99.9
	3.90	3.79	97.2
	12.5	12.52	100.2
Ammeline	0.78	0.90	115.5
	1.25	1.22	97.2
	3.90	3.78	97.0
	12.5	12.52	100.3
Cyanuric acid	0.78	0.67	86.1
	1.25	1.40	111.9
	3.90	3.85	98.8
	12.5	12.51	100.1

Table 5. Calibration Data for Quantification of Melamine and its Derivatives in Soy Meal Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.76	97.7
	1.25	1.20	96.3
	3.90	3.98	102.2
	12.5	12.48	99.8
Ammelide	0.78	0.72	92.9
	1.25	1.18	94.2
	3.90	4.07	104.4
	12.5	12.46	99.7
Ammeline	0.78	0.81	103.7
	1.25	1.22	97.9
	3.90	3.90	99.9
	12.5	12.50	100.0
Cyanuric acid	0.78	0.71	91.3
	1.25	1.22	94.5
	3.90	4.49	115.1
	12.5	12.01	96.1

Confirmation

The identification point system was developed by EU scientists to define an acceptable procedure for scientifically confirming the presence of regulated substances. The more identification points provided by the analytical method, the more certain is the confirmation of the compound. Three points are required for compounds with an MRL. When no MRL can be defined because of the toxicity of the compound, it is banned at all levels. These compounds require four identification points. While four ions need to be monitored by GC/MS to provide four identification points, only two SRM transitions need to be monitored when using triple quadrupole GC/MS/MS. Analysis of melamine and its analogs was performed using at least two SRM transitions for each compound on the triple quadrupole GC/MS system to provide screening and positive confirmation in the same run.

Figures 7 and 8 illustrate the quantifying and qualifying transition profiles for the GC separation of each of the four compounds in both baby formula and soy meal. In each case the qualifying transitions have been normalized to the quantifying transition in order to better illustrate the identical peak shape obtained from both. These transitions therefore provide a positive confirmation of each of the four compounds in each of the sample matrices.

Conclusions

The FDA GC/MS method for screening for melamine, ammelide, ammeline, and cyanuric acid has been modified for use on the Agilent Triple Quadrupole GC/MS system in order to provide screening, quantification and confirmation in one short run. This method does not require any changes in extraction or derivatization procedures, and cycle time is about 15 minutes. In addition, this method meets the new FDA requirement for sensitivity of 0.25 ppm, and it demonstrates excellent linearity of quantification up to 2.5 ppm. Accuracy of quantification is greater than 97% and two SRM transitions for each of the four compounds have been demonstrated in order to provide sufficient identification points for a positive confirmation.

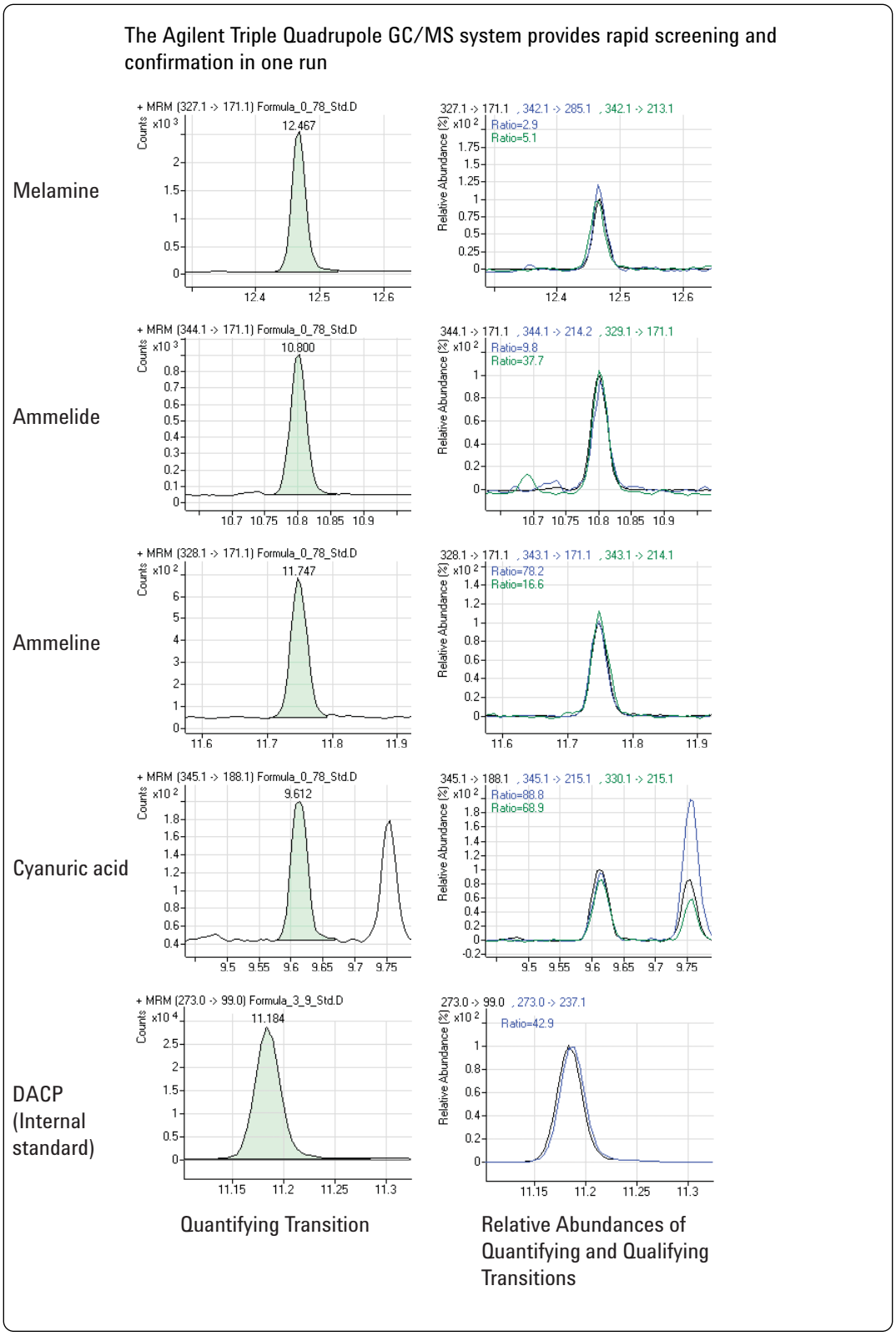

Figure 7. The Agilent Triple Quadrupole GC/MS system provides confirmation and screening in one run: quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in baby formula.

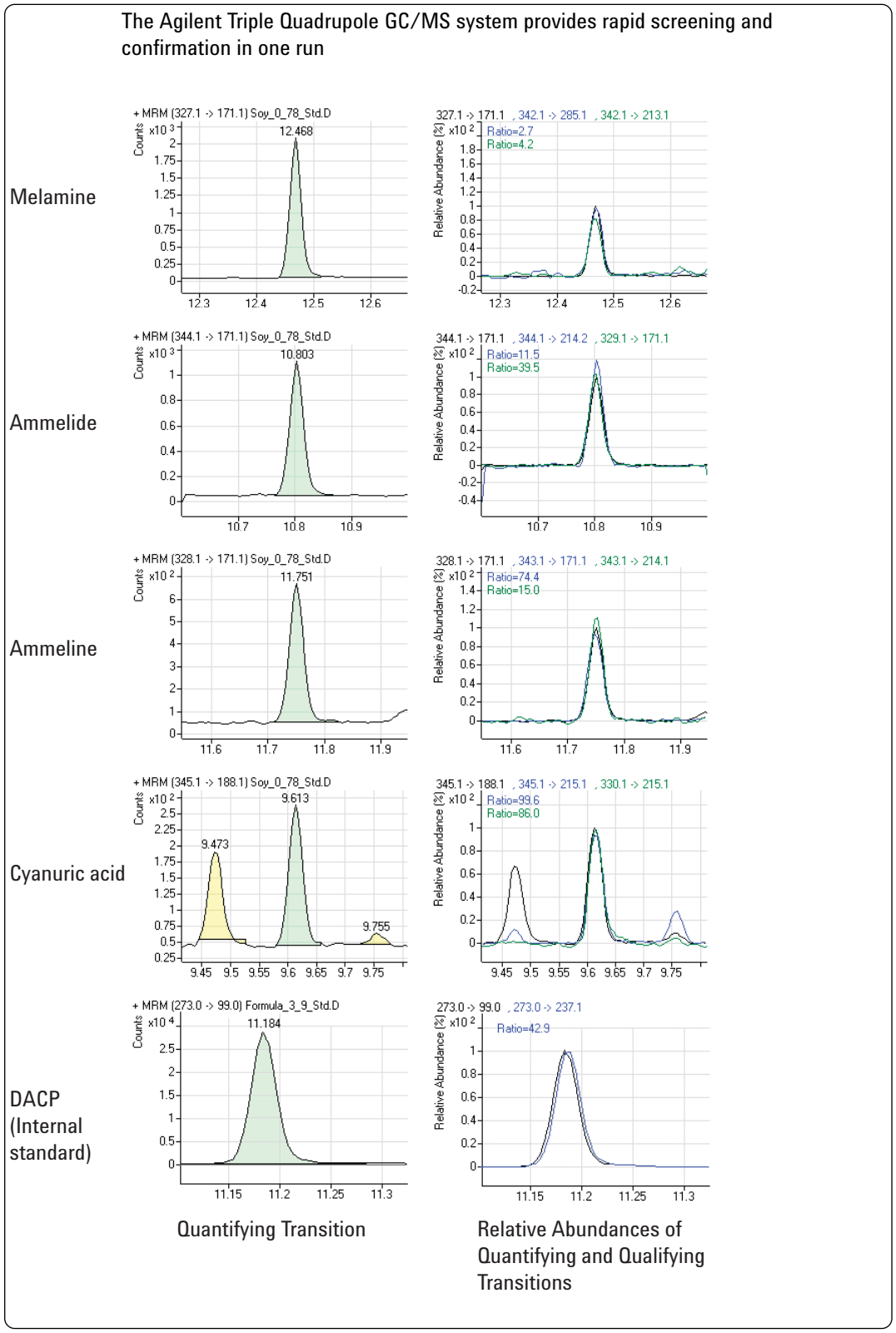

Figure 8. Quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in soy meal.

Acknowledgement

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References

1. U.S. Food and Drug Administration, "GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide, and Cyanuric Acid," LIB No. 4423, Volume 4, October 2008.
2. H. Prest, C. Foucault and Y. Aubut, "Capillary Flow Technology for GC/MS: Efficacy of the Simple Tee Configuration for Robust Analysis Using Rapid Backflushing for Matrix Elimination," Agilent Technologies publication 5989-9359EN.
3. H. Prest, Capillary Flow Technology for GC/MS: "A Simple Tee Configuration for Analysis at Trace Concentrations with Rapid Backflushing for Matrix Elimination," Agilent Technologies publication 5989-8664EN.

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