APPLICATION NOTE 73901

Accurate method for lactose determination in “lactose-free” and “low-lactose” labeled food products

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Goal

Develop an easy and direct method to confirm “lactose-free” or “low-lactose” labeling in complex food matrices using HPIC and HPAE-PAD technologies

Introduction

Lactose, commonly present in dairy products, is also found in processed food products like deli meats, sausages, and food additives. Lactose intolerance is the most common form of food intolerance. In addition, many people are lactase non-persistent. Lactase is an enzyme that hydrolyses lactose into glucose and galactose,[1] and lactase non-persistence is an autosomal recessive trait that leads to lactose maldigestion[2]. Lactase non-persistent individuals show a decline of lactase production after weaning. Consequently, lactose-intolerant individuals have varying degrees of lactose intolerance as adults. They have difficulties digesting lactose-containing products[3,4]. In Asia, nearly one hundred percent of the population is lactose intolerant. The European situation is drastically different, where lactose intolerance varies from 8% to 70% from the North to the South. Due to the increased presence of lactose in many food formulations, the need for an efficient analysis has increased at food laboratories.

Carbohydrates are very hydrophilic and have no chromophore. Due to these specific chemical properties, carbohydrate analysis remains a challenge. Various technical approaches have been used for lactose determination in food products: an enzymatic method, spectrophotometric assays, gas chromatography, capillary electrophoresis, and biosensors. The common approach is liquid chromatography coupled with a refractive index detector. However, this conventional methodology fails for samples with very low lactose content. To find a solution for these samples, we evaluated a new Thermo Scientific™ Dionex™ CarboPac™ column selectivity coupled with a Thermo Scientific™ Dionex™ ICS-6000 HPIC™ ion chromatography system with an eluent generator to simplify the analytical process. High pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) remains the best-in-class technique for determining mono-, di-, and oligosaccharides.[5,6] HPAE-PAD methods have been published for "lactose-free" analysis of dairy products.[7,8] In this study, we designed a lactose determination to confirm "lactose-free" (< 0.01% w/w) and "low-lactose" (<1% w/w) allegations in other complex food samples using 4 µm column technology and an appropriate sample preparation.

Experimental

Equipment

- Dionex ICS-6000 HPIC system:
  - Dionex ICS-6000 DP Quat / Iso Pump module (P/N 21181.60010)
  - Dionex ICS-6000 SP Quat Pump module (P/N 21181.60002)
  - Dionex ICS-6000 EG Module, (P/N 22181-60019)—Degas Unit for EG (SB/MB), (P/N 075522)
  - Dionex ICS-6000 DC 2 V Detector/Chromatography module (P/N 22181-60049)
  - Electrochemical Detector (P/N 072043)
  - Thermo Scientific™ Viper™ PEEK .007 ID. 5.5" [140 mm] (P/N 088806) used as a sample loop (internal volume 3.5 µL)
  - Thermo Scientific™ Dionex™ AS-AP Autosampler and sample tray cooling, 250 µL sample syringe (P/N 074925)
  - Dionex ICS-6000 ED Electrochemical Detector Cell (P/N 072044)
  - Valve assembly, includes one 2-way 6-port valve and mounting hardware (P/N 074123)
  - Thermo Scientific™ Dionex™ EO Eluent Organizer Tray with two 2 L bottles × 2 (P/N 072057)
  - Thermo Scientific™ Dionex™ EO Regulator Accessory and Stand (P/N AAA-074423)
  - IC PEEK Viper Fitting Kit for Dionex ICS-6000 with ED (P/N 088804)
  - Dionex ICS-6000 EG Eluent Generator Kit (P/N 075522)
  - Thermo Scientific™ PEEK Tee 10.32, 0.02 ID (P/N P-727)
- Thermo Scientific™ Megafuge™ 16R Centrifuge (P/N 75004270)
- Thermo Scientific™ LP Vortex Mixer (P/N 15298834)
- Fisherbrand™ Analytical Balance (Model FAS224)
- Thermo Scientific™ Barnstead™ Smart2Pure™ water purification system (model Smart2pure Pro UV/UF 16LPH)
- Thermo Scientific™ F1-ClipTip™ Variable Volume Single Channel Pipette 2-20 µL (P/N 4641180N)
- Thermo Scientific™ F1-ClipTip™ Variable Volume Single Channel Pipette 20-200 µL (P/N 4641210N)
- Thermo Scientific™ F1-ClipTip™ Variable Volume Single Channel Pipette 100-1000 µL (P/N 4641230N)

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.3

Reagents

- Methanol, Optima™ LC/MS Grade, Fisher Chemical™ (10031094)
- Sodium azide, white powder, Fisher BioReagents (10677243)
- Sodium hydroxide 50% w/w 100 mL Fisher Chemical™ (S/4930/05)
- Dionex sodium acetate salt, electrochemical grade, Thermo Scientific™ Dionex™ (059326)
- Alpha-D-lactose, monohydrate, ACS reagent, ACROS Organics™ (412975000)
- L(-)Fucose ACROS Organics™ (225880050)
- Deionized (DI) water, Type 1 reagent grade, 18 MΩ·cm resistivity or better

Consumables

- Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm Separator Column, 2 mm × 150 mm (P/N 088954)
- Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm Guard Column, 2 × 30 mm (P/N 088955)
- Gold working electrode with 1 mil gasket (P/N 061875)
- Reference electrode pH, Ag/AgCl (P/N 061879)
- Greiner Bio-One™ CellStar™ Test Tubes 50 mL Polypropylene Fisher Scientific (Cat No 10480623)
- Thermo Scientific™ Titan3™ Syringe Filters, 17 mm PVDF membrane (P/N 44513-PV)
- FisherBrand™ 1 mL plastic syringe PP (P/N 14-955-456)
- Thermo Scientific™ Vial Kit 1.5 mL glass with caps and septa, 100 each (P/N 055427)
- Thermo Scientific™ Dionex™ OnGuard™ II A cartridges (1 cc) (P/N 057091)
- Thermo Scientific™ Dionex™ CR-ATC 600 Continuously Regenerated Trap Column (P/N 088662)
- Thermo Scientific™ Dionex™ EGC 500 KOH cartridge (P/N 075778)

Instrument method

The method setup is slightly different from conventional setups (Figure 1). It is based on a Dionex ICS-6000 HPIC system with three separate pumps. Pump 1 supplies the eluent generator (EG) with DI water to generate the hydroxide gradient to separate lactose from the other sample components in less than 8 min. Pump 2 delivers DI water for the Dionex CR-ATC. By supplying the regeneration side of the Dionex CR-ATC instead of using eluent recycling as is typically done, we avoid contamination of the Dionex CR-ATC by the sample matrix and thus increase its lifetime. The third pump delivers a rinsing solution that is mixed with eluent using a tee. Mixed solution is pumped into the column to elute column contaminants that cannot be removed with only hydroxide. During this step, the flow rate from pump 1 is decreased to ensure the column pressure is below the maximum operating pressure (5,000 psi). After this step, pump 3 is stopped, and the column is flushed with starting eluent (8 mM) for 9 min at 200 µL/min (Tables 2b and 3).

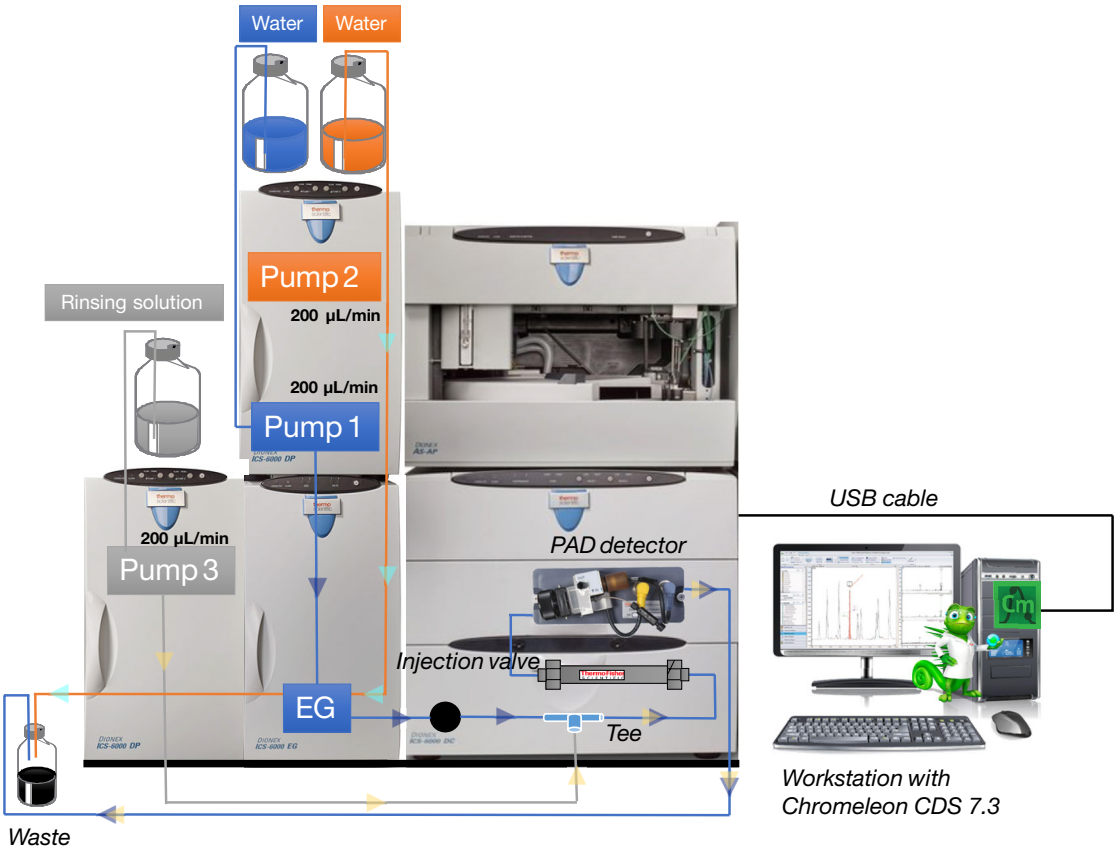


Figure 1. instrument setup

Table 1. IC conditions

	Type
Column:	Dionex CarboPac PA210-Fast-4µm, 2 × 150 mm with guard column
Eluent:	KOH gradient from 8 to 100 mM generated by a Dionex ICS-6000 EG Eluent Generator (For details, see Table 2a)
Eluent source:	Dionex EGC 500 KOH cartridge (P/N 075778), Dionex™ CR-ATC 600 trap column (P/N 088662), high pressure degasser module (Dionex CR-ATC 600 remains continuously regenerated using water at 0.2 mL/min)
Flow rate (eluent):	Table 2b
Rinsing solution:	0.1 M Sodium acetate in 0.1 M sodium hydroxide
Flow rate (rinsing solution):	Table 3
Injection volume:	3.5 µL
Column temperature:	45 °C
Detection:	Pulsed amperometric detection at 20 °C Reference electrode Ag/AgCl Waveform used—Table 4

Table 2a. KOH gradient generated by the Dionex ICS-6000 EG module

Time (min)	Concentration (mM)
0	8.00
0.1	8.00
8	10.13
8	8.00
19	8.00

Table 2b. Flow rate of the eluent pump (Pump 1)

Time (min)	Flow rate (mL/min)
0	0.200
7.75	0.200
7.75	0.025
10	0.025
10.25	0.200
19	0.200

Table 3. Flow rate of the rinsing pump (Pump 3)

Time (min)	Flow rate (mL/min)
0	0.0
7.75	0.2
10	0.2
10	0.0
19	0.0

Table 4. PAD waveform (vs. Ag/AgCl)

Time (ms)	Voltage (V)
0	0.1
200	0.1, Start integration
400	0.1, Stop integration
410	-2.0
420	-2.0
430	0.6
440	-0.1
500	-0.1

Rinsing solution preparation

To prepare 1 L of the rinsing solution, dissolve 8.20 g of sodium acetate powder in approximately 800 mL of DI water. Filter this solution through a 0.2 µm nylon filter to remove particles introduced by the sodium acetate. After filtration, transfer the solution to a 1 L volumetric plastic flask, add 5.2 mL of NaOH (w (NaOH) = 50%), and bring to volume with DI water. Immediately transfer this solution to a plastic eluent bottle on the HPAE-PAD system and blanket with nitrogen at 2 psi (14 kPa).

Sample preparation

In this work, four different samples were analyzed: a low-lactose processed food, Morteau sausage, dry-cured ham, and a food additive powder. Sample preparation is based on previous studies performed only with dairy products. Those studies have shown success using Carrez solutions and Dionex OnGuard sample pre-treatments before injection[6,7]. In this study, we have replaced Carrez precipitation with solvent precipitation using methanol. For salted meats, methanol-based clarification is slightly better than Carrez precipitation (Figure 2). Other approaches, such as using an azide or hydroxide solution, yield no precipitation even after centrifugation at low temperature. Moreover, lactose retention time stability was improved using methanol sample preparation.

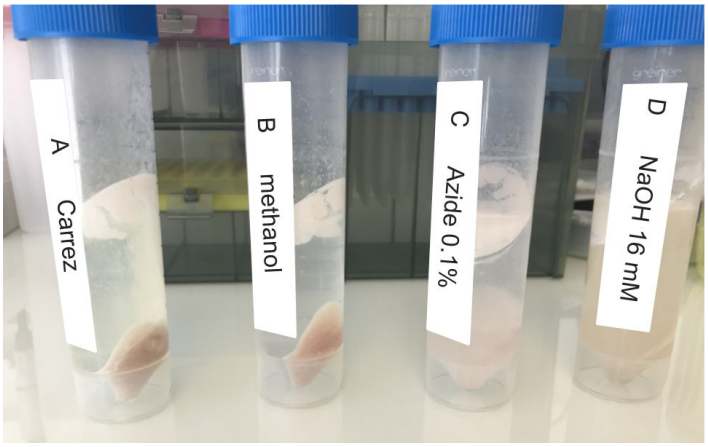


Figure 2. Dry-cured ham clarification using Carrez, methanol, azide, and sodium hydroxide solutions

Diluent preparation: Twenty-five milligrams of sodium azide were dissolved in DI water in a 250 mL volumetric flask. The diluent solution, used as a bacterial preservative, did not affect the separation and quantification of sugar as already reported by Ting-Jang et al.[9]

Lactose 200 ppm stock solution: Weigh 10 mg of lactose powder in a 50 mL polypropylene tube and dissolve in diluent solution using a magnetic stirrer at room temperature.

Internal standard 200 ppm stock solution: Weigh 10 mg of fucose powder in a 50 mL polypropylene tube and dissolve in diluent solution using a magnetic stirrer at room temperature.

The ready-to-use stock solutions and working standard solutions were stored at 4 °C and remained stable for several weeks.

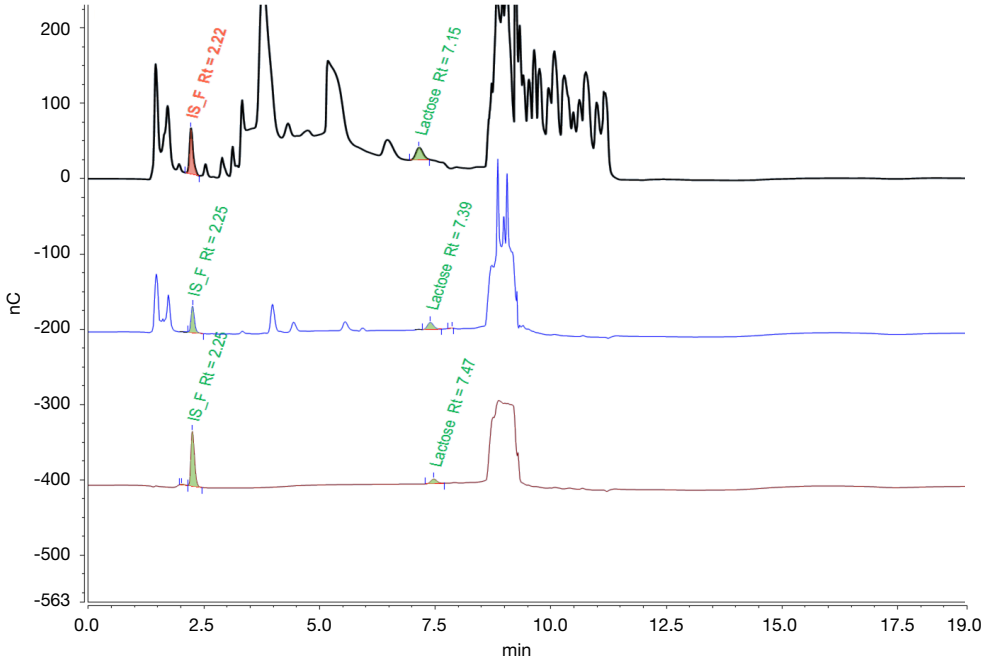
Weigh 1 g of raw material into a 50 mL polypropylene tube. Add 10 mL of methanol. Shake vigorously. Add 2 mL of internal standard stock solution.

- For unspiked sample, add 13 mL of diluent solution
- For spiked sample, add 600 or 2000 µL of lactose stock solution and add 12.4 mL and 11 mL of diluent, respectively.

Shake 30 min using an orbital stirrer (300 rpm). Centrifuge at 6000 g for 15 min. Pass 3 mL of supernatant through a Thermo Scientific™ Dionex™ OnGuard™ II A cartridge (previously conditioned with 10 mL of DI water) and a 0.45 µm PVDF filter. Discard the first 2 mL to waste and collect 1 mL. Dilute the solution two-fold with diluent solution directly in the injection vial. Vortex the solution for a few seconds and place the tube into the AS-AP autosampler.

Results and discussion

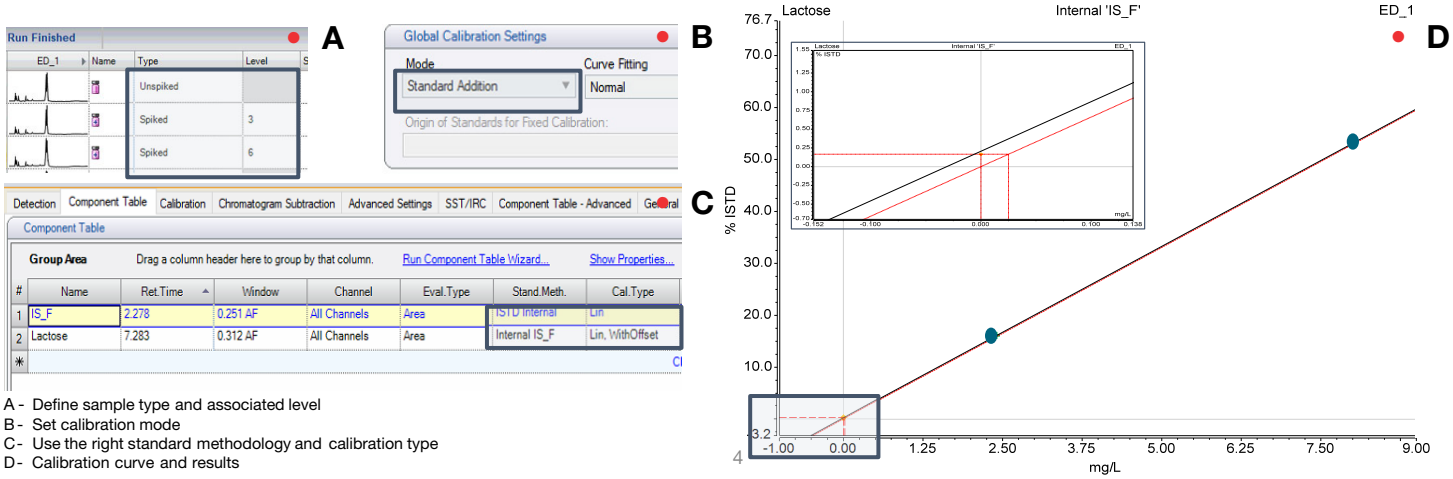
Hydroxide gradient elution, combined with a high column temperature, allows lactose separation from matrix compounds in 19 min injection to injection (Figure 3). Using eluent generation, we eliminate the need to prepare hydroxide-based eluent, and due to the high purity of the eluent, we increase gradient reproducibility. The analytical format of the eluent generator allows up to 100 mM high purity potassium hydroxide generation. This hydroxide concentration is unable to elute compounds strongly bound to the column. Therefore, we used an additional pump to add strong eluent in the fluidic pathway. The high eluent strength of the rinsing solution washes the column before the next sample injection. A significant concentration of contaminants in the food additive powder sample are eluted during this rinsing step (Figure 3). Under these conditions, lactose retention time remains stable for a dedicated matrix. Depending on the tested matrix, lactose retention time varies from 7.17 to 7.47 min. Matrix compounds can also affect lactose's electrochemical response. To compensate for this potential effect, we introduced an internal standard: IS_F (Internal Standard Fucose). To compensate for measurement errors due to sample preparation, injection, and possible effect of the sample matrix, we used the standard addition calibration mode coupled with internal standard correction. Figure 4 illustrates the software process (from A to D) to set up this processing method to measure low lactose concentrations in samples.



Columns:	Dionex™ Carbopac PA210-Fast-4µm 2 × 150 mm with guard
Eluent:	KOH gradient (for details see Table 2a)
Eluent source:	Dionex EGC 500 KOH cartridge, Dionex CR-ATC 600 trap column
Flow rate:	Table 2b
Inj. vol.:	3.5 µL
Oven temp.:	45 °C
Detector:	PAD
Sample:	Black trace = food additive powder Blue trace = dry cured ham Brown trace = standard in water
Peak: Lactose	Retention time (min) See chromatograms

- * IS_F fucose used as internal standard

Figure 3. Matrix effect on lactose retention time shift



A - Define sample type and associated level
B - Set calibration mode
C - Use the right standard methodology and calibration type
D - Calibration curve and results

Figure 4. Standard addition calibration process using Chromeleon 7.3 software

Routine analysis is based on three injections per sample: one native and two spiked at two concentrations. Before determining the amounts to spike into samples, the linearity of the calibration curve was checked. Five increasing volumes from 200 to 1000 µL of lactose stock solution were added to prepared samples of Morteau sausage (A) and dry-cured ham (B). Lactose calibration curve linearities were good with both coefficients of determination greater than 0.99 (Figure 5).

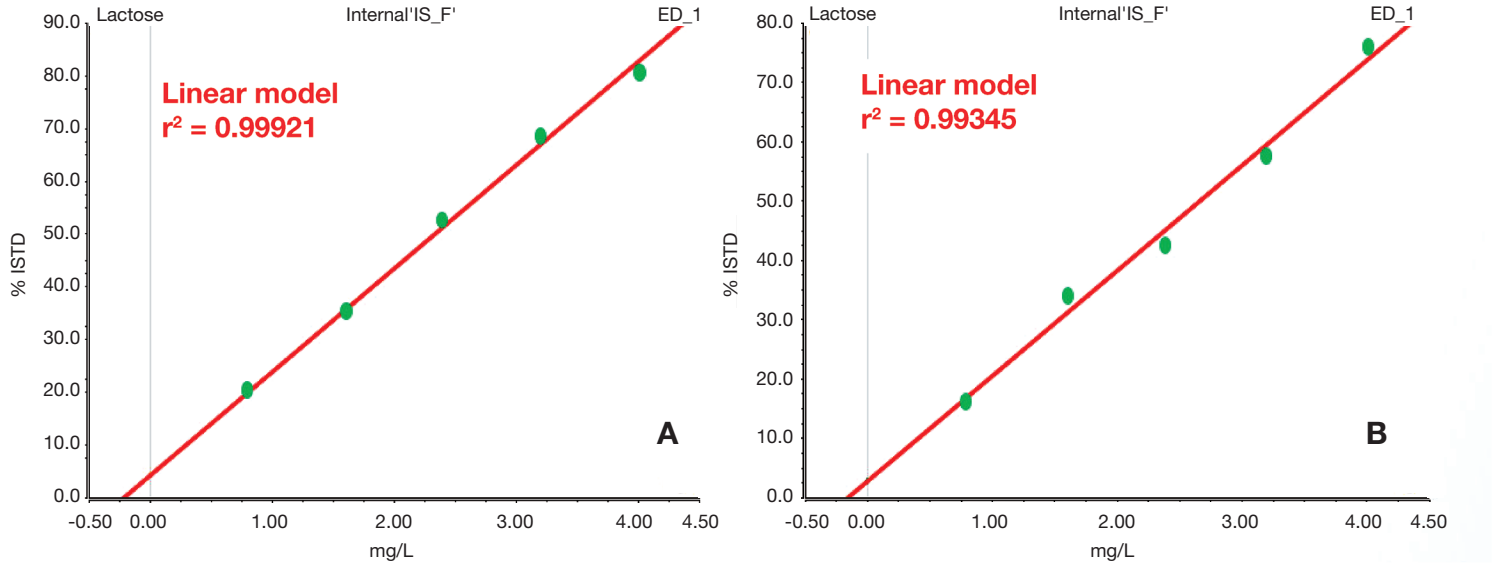


Figure 5. Lactose calibration curves obtained in Morteau sausage (A) and dry-cured ham samples (B)

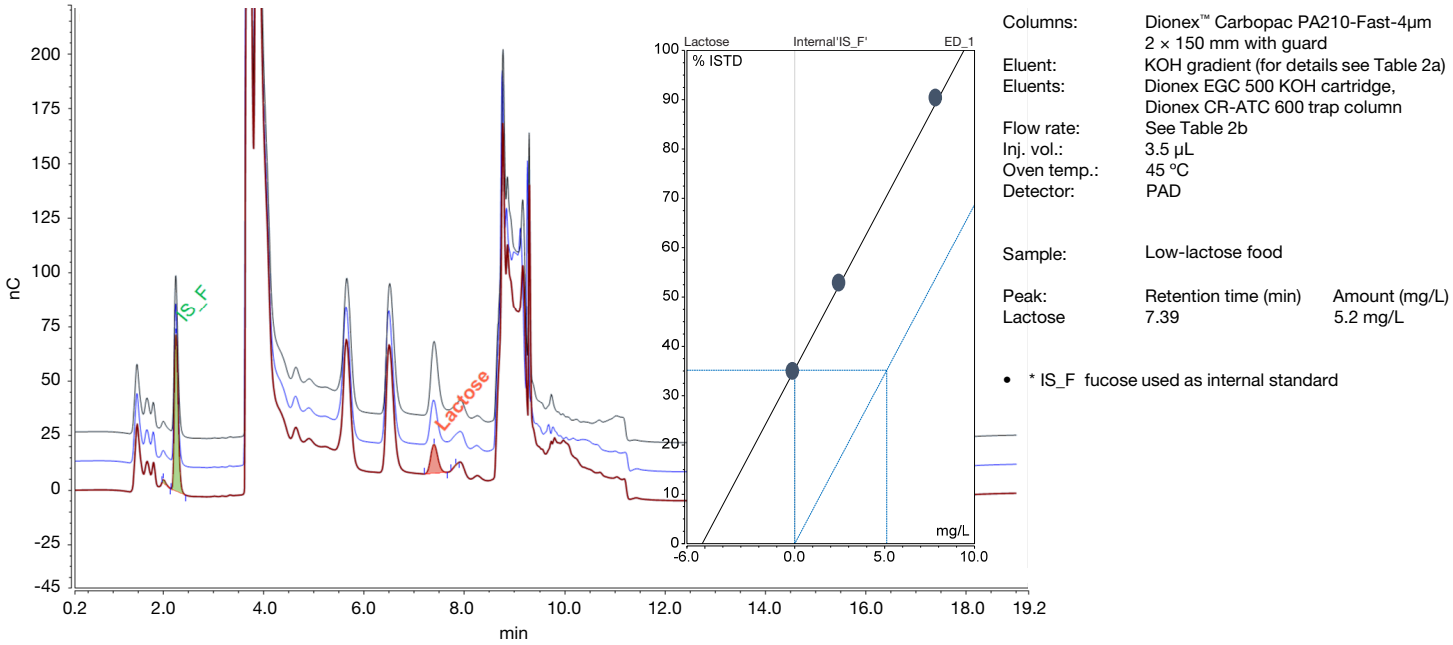


Figure 6. Overlay of chromatograms obtained after 3.5 µL injection of low lactose food (brown trace = unspiked, blue trace = spiked 2.4 mg/L, and black trace = spiked 8 mg/L)

Figure 6 shows excellent retention time stability. This simplifies interpretation and reduces reprocessing time. In our experience, reliability is drastically improved in comparison with previous work that used other Dionex CarboPac columns, HPAE-PAD technology, and external calibration. No common limit values have been defined so far at the EU level for the lactose content of low-lactose and lactose-free food products. For example, for a lactose-free allegation, national limits range from 100 mg lactose per 100 g (0.1%) in Germany to 10 mg lactose per 100 g (0.010%) in France. In contrast, the "low-lactose" definition has a more broadly accepted value of 1 g lactose per 100 g (1%). Quantification of lactose at low levels is easier using the standard addition calibration mode. The amount of lactose calculated in the low-lactose content food shown in Figure 6 is 5.2 mg/L. This lactose amount of 0.026% (26 mg/100 g) is between the French lactose-free limit of 0.01% and low-lactose content limit of 1%. Using this sample preparation, the lactose amount in the autosampler vial must be lower than 2 mg/L to declare the sample a "lactose-free" product.

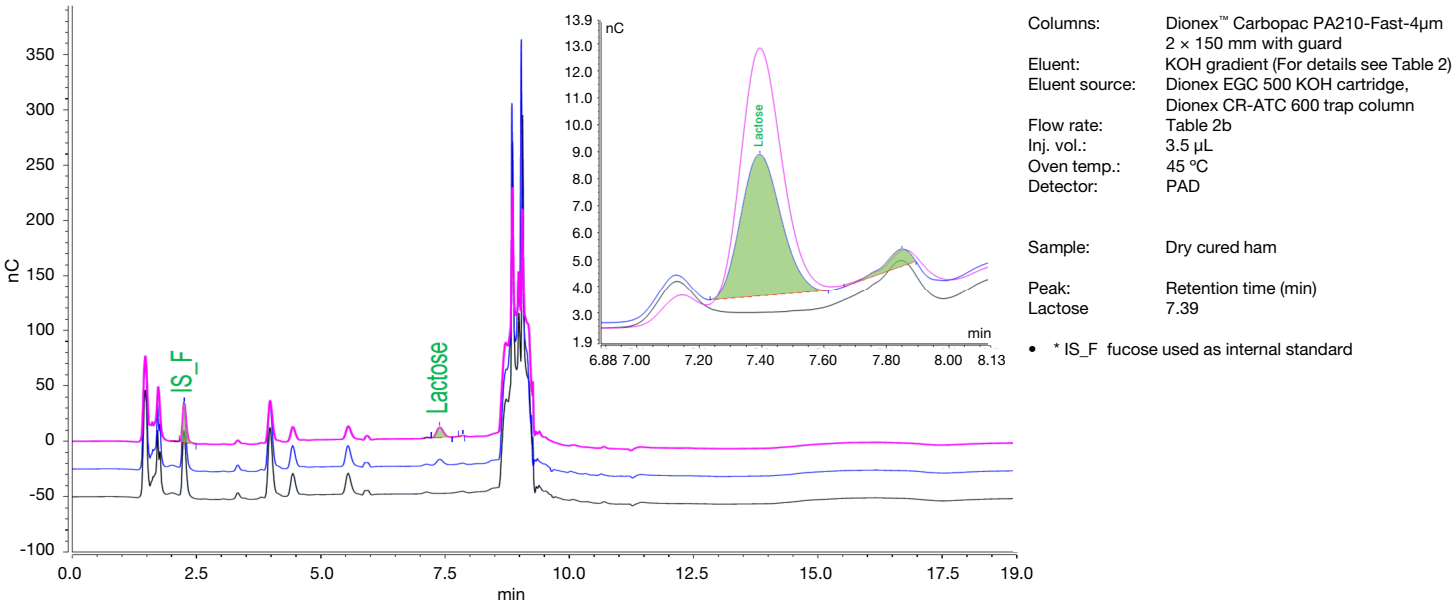


Figure 7. Overlay of chromatograms obtained after 3.5 µL injection of dry-cured ham extracts (black trace = unspiked, blue trace = spiked 2.4 mg/L, and pink trace = spiked 8 mg/L)

The new setup allows the confident confirmation of a lactose-free allegation in complex food samples. As shown in Figure 7, lactose can be detected below the French regulatory limit using PAD; however, detection specificity in complex matrices remains insufficient. That is why a gradient was developed to separate lactose from two interferents. Depending on the matrix, slight gradient modifications can be applied to adjust lactose retention time. Figures 7 and 8 show chromatograms of native and spiked samples with two different concentrations of lactose, dry-cured ham and food additive extracts, respectively. Chromatograms of the native samples confirm the absence of lactose in each sample as the 2.4 mg/L spike is just above the French limit of 0.01% and well below the German limit of 0.1%.

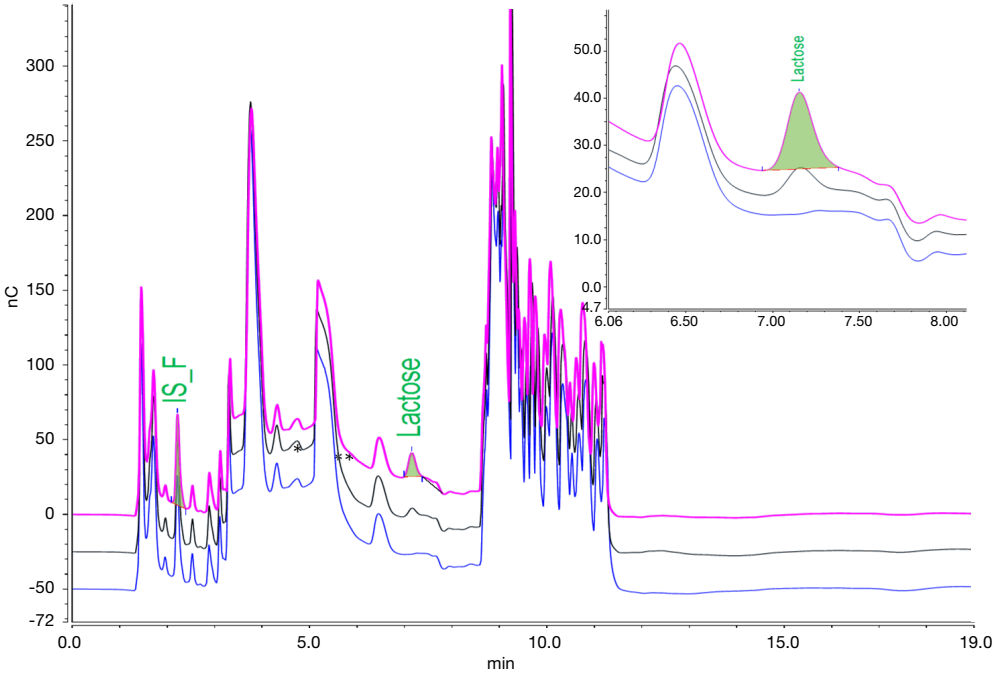
This lack of lactose in this dry-cured ham was confirmed by an independent laboratory using its own technique. This lactose-free matrix was perfect to evaluate recovery of lactose. Ten sample preparations of this matrix were fortified with a known amount of lactose prior to sample preparation. Recoveries were calculated and reported in Table 5. Recoveries of lactose were 98 to 105%.

Table 5. Recovery of lactose in dry-cured ham extracts spiked with 3.2 mg/L of lactose

Repetition	Recovery (%)
# 1	104
# 2	104
# 3	105
# 4	103
# 5	104
# 6	102
# 7	98.2
# 8	97.5
# 9	98.0
# 10	102
Average	**102**
± SD	2.61

Conclusion

This new IC method that includes sample preparation and four-micron column technology allows lactose determinations in non-dairy food samples labeled as low-lactose and lactose-free. The described instrumental setup includes a rinsing step to ensure good retention time stability in these complex samples. The method was shown to confirm “lactose-free” designations of foods sold in France that must conform to a 0.01% limit.



Columns:	Dionex™ Carbopac PA210-Fast-4µm 2 × 150 mm with guard
Eluent:	KOH gradient (for details see Table 2a)
Eluent source:	Dionex EGC 500 KOH cartridge, Dionex CR-ATC 600 trap column
Flow rate:	Table 2b
Inj. vol.:	3.5 µL
Oven temp.:	45 °C
Detector:	PAD
Sample:	Food additive powder
Peak:	Retention time (min)
Lactose	7.15

- * IS_F fucose used as internal standard

Figure 8. Overlay of chromatograms obtained after 3.5 µL injection of food additive powder (black trace = unspiked, blue trace = spiked 2.4 mg/L, and pink trace = spiked 8 mg/L)

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