Prediction of stable isotopes and fatty acids in subcutaneous fat of Iberian pigs by means of NIR: A comparison between benchtop and portable systems

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ABSTRACT

The potential of a portable Near Infrared spectrophotometer compared with that of NIR benchtop equipment is assessed to determine the $^{13}C/^{12}C$ relationship of stable isotopes and the fatty acid content. 105 samples of subcutaneous fat of Iberian pigs collected at the time of their slaughter have been analyzed. The analysis of stable isotopes and gas chromatography were the methods of reference used. The samples were analyzed without prior handling (portable and benchtop NIR) and after extracting the fat (benchtop NIR). The results show that with the portable equipment it is possible to determine $\delta^{13}C$ (‰), 12 fatty acids, and 5 summations of fatty acids (SFA, MUFA, PUFA, w3, and w6), while with the benchtop NIR equipment it is possible to measure $\delta^{13}C$ (‰), 16 fatty acids, and the 5 summationsof fatty acids. The correlation coefficients of the portable equipment were slightly lower than those of the NIR benchtop equipment.

1. Introduction

The products deriving from the Iberian pig (ham and shoulder) are highly appreciated by consumers for their organoleptic and gastronomic quality. Although this quality depends on the genetics of the pig, what it eats, and the technology used in the production [1,2], various authors have shown that the factor with the greatest influence is its food [3]. Therefore, depending on whether the diet is based onfodder or on montanera (the consumption of acorns and grass by free grazing) a significant change occurs in the profile of the fatty acids of the fat of Iberian pigs [4]. The industries of the Iberian sector have implemented programs for controlling the fatty acids of subcutaneous fat as an indicator of the diet followed by the animal during the montanera. The importance for the sector lies in the fact that the fatty acid composition has a considerable influence on the sensory profile [5]. However, the existence of fraudulent practices such as complementing the final fattening diet of Iberian pigs with commercial foods which imitate the composition of montanera means that it is important to determine the composition of fatty acids rapidly and reliably. The analysis of the profile of fatty acids (FA) has traditionally been carried out by means of gas chromatography; however, near infrared reflectance spectroscopy (NIRS) has shown its potential for predicting the composition of fatty acids in adipose tissue, extracted fat, or rendered fat of Iberian pigs [6–8].

On the other hand, the carbon isotope composition can also provide information on the animal feed. The $\delta^{13}C$ value of cattle was found to be highly dependent on their diet composition, particularly with regard to the proportion of C4 and C3 plant material [9]. By using the stable isotopes of C, H, N, O, and S and isotopes of Sr and B in different food products of both animal and plant origin it is possible to ascertain the origin, diet, and production system [10,11]. In the case of the Iberian pig (a monocavitary animal), the $^{13}C/^{12}C$ isotope relationship expressed as $\delta^{13}C$ (‰) shown in its subcutaneous fat allows the reconstruction of the diet followed by the animal. Various studies have therefore shown that it is possible to distinguish Iberian pigs fed on acorns from those fed another kind of diet (mixed and raised) [12–14]. Moreover, stable isotopes of carbon ($\delta^{13}C$) and sulfur ($\delta^{34}S$) in liver samples allow the distinguishing of the diet received by the pigs during fattening (acorns or

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fodder) and also of their breed (Iberian or white). The analysis of stable isotopes of H, C, N, O, and S in cured hams from Italy and Spain with different genetics and production systems has allowed the distinguishing of geographical origin and is considered to be a potentially useful method for determining also the production system [15]. The use of the relationship of $^{87}Sr/^{86}Sr$ isotopes of strontium and the analysis of 34 trace elements allows the distinguishing of the geographical origin (Spain or Italy) of the hams studied (Iberian, Bayona, Parma, and San Daniele) and the processing practices with the salt content, although it is considered that more detailed study is necessary [16]. It should be emphasized that the analysis of stable isotopes of carbon is an expensive method which is hard to access for researchers and Iberian pig companies.

Given the complexity of the processes related to the Iberian pig, rapid and decisive methods are sought. In this sense near infrared spectroscopy (NIR) has been shown to be a reliable, cheap, and rapid analytical method. Within the products deriving from the pig both in muscle and in fat, infrared spectroscopy has been used to determine the fatty acid profile [8,17,18], in the authentication of the fattening diet of the pigs [19–21], and combined with a PLS analysis to predict defects in serrano ham among others [22,23]. Studies of NIR technology for the prediction of stable isotopes have not been found. The recent development of portable devices which allow the establishing of NIRS technology in industrial environments means that they have great potential as an in situ control tool and for routine monitoring along value chains in real time. Examples of this include applications to agrofood products [24], dairy products and cheeses [25,26], soils [27], or delicate revisions of spectroscopy techniques with portable instruments [28]. In the matter which concerns us, portable NIRs are being used with great potential in the meat sector in aspects related to product quality and safe [29,30]. In the sector of the Iberian pig in particular, portable instruments coupled to optic fiber have been used to calibrate four of the main fatty acids in live animals, in carcasses at the abattoir, in subcutaneous fat (with and without skin), andin transverse sections [31]. However, the equipment has its limitations regarding cost, time, weight, and a lack of sensitivity.

Other portable equipment, the so called micro-electro-mechanical system (MEMS)-NIRS, has been applied to Iberian pig carcasses at the abattoir in order to classify the animals according to their food type ("Acorn", "Mixed Acorn and Fodder", and "Fodder") [20]. More recently it has been applied to fresh meat and subcutaneous fat to achieve a discrimination rate of 75% according to the four official categories for Iberian pig carcasses (Black, Red, Green, or White Label) [32]. When the results obtained by the (MEMS)-NIRS equipment, which operates in the area of the 1600–2400 nm spectrum with data every 8 nm, are compared with those obtained by high-resolution NIRS monochromators, which operate in the 400–2500 nm margin with data every 2 nm, it can be concluded that the results for the four main fatty acids are acceptable for the Iberian pork industry [33]. Despite these results, some studies Pérez-Marín & Garrido-Varo [34]on the potential of NIRS for the in situ analysis of ham from the Iberian pig, and on the use of sensors to ensure robust traceability, indicate that these are matters which still need to be debated. This study compares the results of the prediction of stable isotopes and of up to 16 fatty acids together with the summation of saturated, monounsaturated, and polyunsaturated fatty acids, w3, and w6 in subcutaneous fat of the Iberian pig, using two systems in order to do so. These are on the one hand portable NIR equipment in the area of the spectrum from 900 to 1700 nm and on the other benchtop NIR equipment with the area of the spectrum from 1100 to 2500 nm.

2. Material and methods

2.1. Samples

105 samples of subcutaneous fat were taken from Iberian pigs at the time of slaughter: 60 samples of 100% pure Iberian and 45 samples of 50% Iberian-Duroc crossbreeds from the 2018–2019 slaughtering season. The animals analyzed had been fed by means of the *montanera* system (consumption of acorns and grass) for periods of between 68 and 120 days. The samples of subcutaneous fat were collected when the animal was slaughtered from the coccyx including the skin along the full depth of the subcutaneous fat of the animal with all the accumulations of fat. Analyses of stable isotopes, the composition of the fatty acid profile, and NIR calibration were carried out on these samples. Las muestras se mantienen congeladas siempre a $-30\,°C$ hasta el momento de la medida.

2.2. Determination of stable isotopes

The skin was removed from the subcutaneous fat sample, the latter was ground up, and some 10 g were homogenized with a Polytron blender. The homogenized samples were kept frozen at $-28°\,C$ until use. The samples were analyzed for ^{13}C using a HCNOS EURO EA 3000 elemental analyzer with a system of combustion at high temperature and a system of dilution connected to a mass spectrometer with a gaseous source in continual flow for the determination of relationships of stable isotopes, model ISOPRIME; made by Micromass. The ^{13}C natural abundance was expressed in ^{13}C units using the international PDB standard: $\delta^{13}C = [(^{13}Rsample/^{13}Rstandard) - 1]1000$, where $^{13}R = {}^{13}C/^{12}C$. The National Bureau of Standards supplies NBS-21 graphite with a $\delta^{13}_{PDB}C$ of 0–28.10‰. It should be noted that the precision (2σ) of CO_2 measurement on a double-beam spectrometer is 0.05.

2.3. Determination of fatty acids

The quantification of fatty acids by means of gaseous chromatography (GC) of the fat extracted from the subcutaneous fat by microwaves (at a power of 1200 W and using position defrosting) [8]. The fatty acids (FA) of all samples were methylated and analyzed by gas chromatography (6890 N gas chromatograph (GC)); Agilent Technologies, Santa Clara, CA), using a SP-2560 capillary column (Supelco, Bellefonte, PA) according to the method described by Lurueña-Martínez et al. [35]. For these samples, 1 μL was injected into the gas chromatograph using a split (20:1). The different FA were identified by their retention times, comparing them with the standard mix (47885-U Supelco, Sigma-Aldrich, Germany) and their contents were calculated using chromatogram peak areas and were expressed as percentages (%) of the total FA (fatty acids) analyzed.

2.4. NIRS measurements

Samples of subcutaneous fat were analyzed by using NIRS instruments: benchtop Foss NIR System 5000 equipment (Hillerod, Denmark) with a standard 1.5 m 210/210 bundle fibre-optic probe (Ref. no R6539-A), and a portable MicroNIR Pro v2.5 equipment (MicroNIR 1700 ES, VIAVI, Santa Rosa, California, USA). The benchtop equipment includes a probe employs with a remote reflectance system and a ceramic plate as reference. The quartz window (with 5 cm × 5 cm surface area) allows the measuring of reflectance in the NIR zone close to 1100–2000 nm. It takes 30 min of warm-up for the portable NIR equipment and 1 h for the benchtop equipment. The measurement of the registry was carried out by applying directly with the probe on intact untreated sample of subcutaneous fat. Extracted subcutaneous fat samples also were analyzed (with total lipid extracts in microwaves). For these liquid samples, 15 μL of extracted fat into cam-lock were used (circular capsules with an optic path length of 0.1 nm) in NIR registers. Measurements were carried out in reflectance mode between 1100 and 2498 nm. The spectra were recorded at intervals of 2 nm, performing 32 scans for both the reference and samples.

Acquisition of spectra data using a portable MicroNIR spectrophotometer was performed with an instrument designed to measure diffuse reflectance in the NIR region of the electromagnetic spectrum. The MicroNIR equipment uses a linear variable filter (LVF) as the dispersing element. As a result of the varying film thickness, the wavelength

transmitted through the filter varies linearly in the direction of the wedge. The LVF is coupled to a linear detector array (128-pixel uncooled InGaAs photodiode array). The ultra-compact spectroscopic engine is coupled with a tungsten lamps diffuse illumination system. The measuring of reflectance in the NIR zone close was 900–1700 nm, and at intervals of 6 nm in the spectra was recorded. The intact subcutaneous fat was measured directly with an adapter coupled to the system NIR. Spectra were recorded using the instrument acquisition software MicroNIR™ Pro v.2.2 (VIAVI, Santa Rosa, California, USA). The portable equipement used a Spectralon® ceramic tile as a white reference (100% reflectance) of politetrafluoroetilen (~99%). A reference measurement was performed on the MicroNIR approximately 20 min after the lamps were turned on and every hour thereafter while performing scans. A 99% diffuse reflectance panel was used for the 100% reference value, and the 0% reference value was taken by leaving the tungsten lampson with an empty support (known as dark Current Scan). Mean temperature on measurement ranged from 21 ± 1 °C. La obtención de los registros NIR tardan 2 minutos. Tratamos de minimizar los errores del muestreo. La razón, el cerdo ibérico refleja en su grasa subcutánea la alimentación recibida a lo largo del tiempo (y con eso la composición en ácidos grasos y $\delta^{13}C$), así los acumulos de grasa más próximo a la piel, corresponden a su última alimentación. Se realizan 3 registros NIR y en cada registro se toman 32 puntos diferentes de la zona de 5cmx5 cm, dando lugar a un espectro medio. El proceso se repite 3 veces y se acepta el espectro medio, si no hay diferencias superiores a H (cte de Mahalanobis). Fig. 1 shows the 3 spectra obtained with the 3 types of register. The diffuse reflectance signal of the NIR spectrum expressed as log (1/R) (R = reflectance). The software used for data manipulation and chemometric analysis was Win ISI 1.50 installed on a Hewlett-Packard Pentium III computer. To develop the models 85 samples were taken in the calibration group and 20 samples in that of external validation, dividing the samples of subcutaneous fat into the two groups at random.

2.5. Statistical analyses for NIR

Sample spectra were pre-processed using a principal component analysis (PCA) to select samples for allocation in either the calibration or validation set and to detect outliers. The number of variables is reduced by principal component analysis (PCA) [36]. A sample with H statistic ≥ 3.0 standardized units from the mean spectrum is defined as a global H outlier and is eliminated from the calibration set. The calibration process was implemented with the spectra of the resulting samples and their chemical data. Using the $T \geq 2.5$ criterion, samples that presented high residual values when they were predicted were eliminated from the set. The statistical parameters of the calibration were obtained for each of the components after removing the samples for spectral (H criterion) or chemical (T criterion) reasons. Anomalous spectra were determined by applying the Mahalanobis distance because the risk of there being mistakes in the equations under practical conditions is very low or practically non-existent when the standardized H-statistic (Mahalanobis distance) is used during the routine analysis of unknown samples. This tells us how different the spectrum of the unknown sample is from the average spectrum in the calibration set. Samples with an H-value greater than three may be considered as not belonging to the population from which the equations were developed. Moreover, using the $T \geq 2.5$ criterion, samples that were different from the population owing to chemical criteria were removed from the set. The quantification of the $\delta^{13}C$ (‰) and fatty acids was performed by the MPLS regression method [37]. MPLS is often more stable and accurate than the standard PLS algorithm. In MPLS the NIR residuals at each wavelength, obtained after each factor has been calculated, are standardized (by dividing the standard deviations of the residuals at each wavelength) before calculating the next factor. All the spectral information (1100–2000 nm) is used when the remote reflectance fiber-optic probe (intact samples) is also used, and 1100 and 2498 nm when the NIR spectrum is obtained with the cam-lock cells and the fat extract, and between 900 and 1700 nm when portable NIR equipment is registered. To minimize the undesired contributions present in the NIR signals different spectral pretreatments were used: derived and smoothed. The effects of scattering were removed using multiplicative scatter correction (MSC), standard normal variate (SNV), detrend (DT), or SNV-DT and combinations of these [38]. Moreover, mathematical treatments, derivatives, gap in derivatives, and
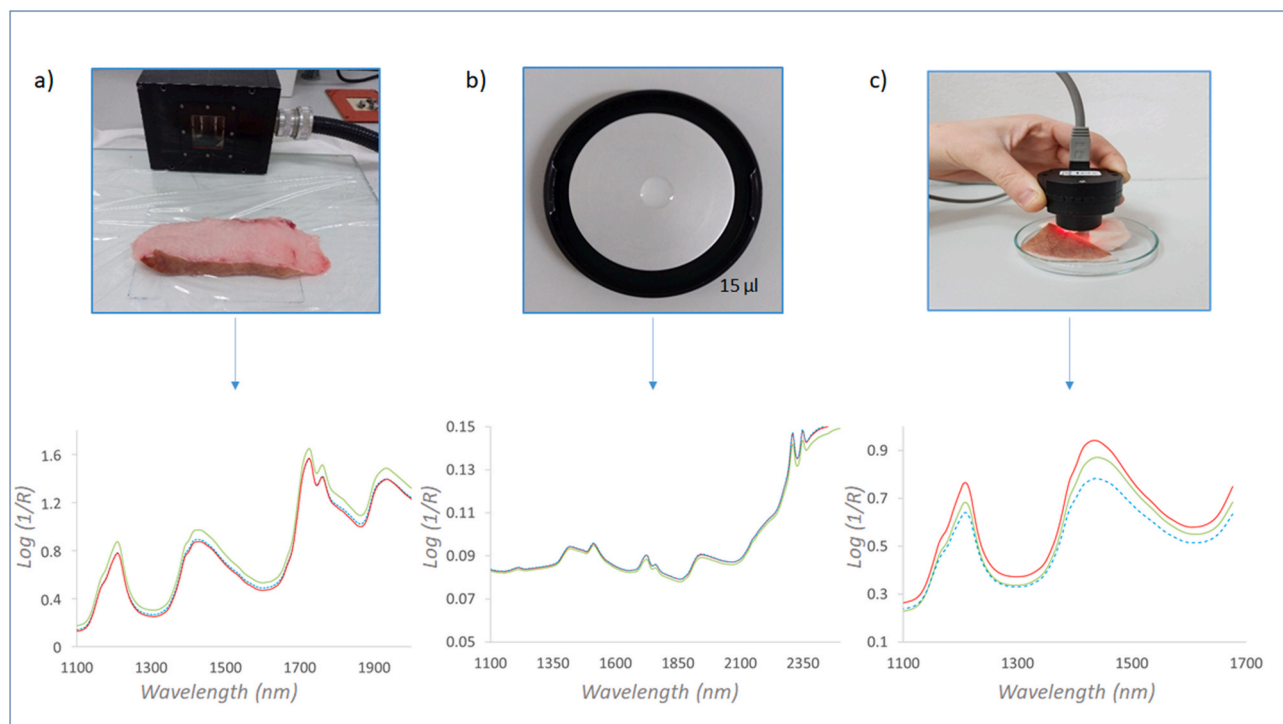

Fig. 1. FNIR measurements: (a) Foss NIR System 5000 with a fibre-optic probe. Samples of intact subcutaneous fat. (b) Foss NIR System 5000 with a Cam-lock probe. Samples of extracted subcutaneous fat and (c) MicroNIR Pro v2.5. Samples of intact subcutaneous fat.

smoothing were carried out. When developing MPLS equations, cross-validation is recommended in order to select the optimum number of factors and to avoid overfitting [39]. For cross-validation the calibration set is divided into several groups. Each group is then validated using calibration developed on the other samples. Finally, validation errors are combined into a standard error of cross-validation (RMSECV). It has been reported that RMSECV is the best single estimate for the prediction capability of the equation. This statistic is similar to the average standard error of prediction (RMSEP) from ten randomly chosen prediction sets [39]. The number of cross-validation groups was 8 for the intact subcutaneous fat samples, 7 for the samples of extracted fat using the register of the benchtop NIR equipment, and 8 for the samples registered with the portable equipment. The ratio of performance to deviation (RPD) is the relation between SD and RMSEC, and it is desired to be larger than 2 for a good calibration [40]. The statistics used to select the best equations were R squared (RSQ, multiple correlation coefficients) and RMSECV.

3. Results and discussion

3.1. Values of reference

The results of the $^{13}C/^{12}C$ relationship of stable isotopes, expressed as $\delta^{13}C$ (‰) of the subcutaneous fat (at the time of slaughter), and of the 30 quantified fatty acids together with the totals of fatty acids by functional groups (SFA, MUFA, PUFA, w3 and w6) are shown in Table 1. It can be appreciated that the components determined show a wide range of variation owing to the fact that the animals spent different periods on the *montanera* and to different breeds. As the time on the *montanera* is

Table 1
Composition of $\delta^{13}C$ and fatty acids in the 105 samples of subcutaneous fat of Iberian pigs used as a reference.

Constituent	Minimun	Maximun	SD
$\delta^{13}C$	−27.46	−24.68	±0.45
C12:0	0.04	0.08	±0.01
C14:0	0.76	1.36	±0.12
C14:1 n5	0.00	0.13	±0.01
C15:0	0.03	0.13	±0.02
C16:0	15.28	22.39	±1.52
C16:1	1.47	2.83	±0.28
C17:0	0.19	0.48	±0.05
C17:1	0.02	0.49	±0.06
C18:0	6.00	12.39	±1.40
C18:1 n9	0.00	0.28	±0.03
C18:1	48.57	58.57	±1.99
C18:1 n7	0.00	2.81	±0.39
C18:2 n6t	0.01	0.16	±0.02
C18:2 n6	6.77	11.92	±1.01
C20:0	0.00	0.22	±0.03
C18:3 n6	0.03	0.29	±0.07
C18:3 n3	0.41	1.37	±0.16
C20:1 n9	1.14	2.17	±0.19
C21:0	0.00	0.17	±0.04
C20:2 n6	0.00	0.06	±0.01
C22:0	0.00	0.05	±0.01
C20:3 n6	0.00	0.05	±0.01
C22:1 n9	0.47	0.84	±0.08
C20:3 n3	0.01	0.05	±0.01
C20:4 n6	0.00	0.12	±0.03
C23:0	0.02	0.12	±0.03
C22:2 n6	0.00	0.29	±0.09
C20:5 n3	0.00	0.27	±0.06
C22:5 n3	0.02	0.19	±0.05
C22:6 n3	0.04	0.09	±0.01
AGS	23.92	36.72	±2.77
AGM	54.25	64.49	±1.91
AGP	8.03	14.06	±1.14
w3	0.76	1.85	±0.20
w6	7.21	12.21	±0.97

SD: Standard Deviation.

Table 2
NIR calibration statistics of $\delta^{13}C$ and fatty acids in subcutaneous fat of Iberian pigs by means of benchtop NIR equipment. Samples of Intact Subcutaneous Fat (ISF) and extracted subcutaneous fat (ESF).

Constituent	N		Treatment		Mean		Est. Min- Est. Max		SD		RMSEC		RPD		RMSEP		RSQ	
	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF	ESF	ISF
$\delta^{13}C$	83	83	Detrend only 1,4,4,1	Standard MSC 2,4,4,1	−26.26	−26.21	−24.95-27.56	−24.85-27.58	0.44	0.46	0.15	0.21	2.90	2.16	0.25	0.22	0.88	0.79
C16:0	83	81	SNV only 2,4,4,1	Detrendonly 2,4,4,1	18.89	18.85	14.42-23.36	14.28-23.42	1.49	1.52	0.36	0.36	4.15	4.22	0.41	0.41	0.94	0.94
C16:1	83	85	Detrend only 2,4,4,1	None 2,4,4,1	2.16	2.16	1.36-2.97	1.35-2.96	0.27	0.27	0.15	0.16	1.79	1.72	0.16	0.16	0.69	0.66
C17:0	84	a	Standard MSC 2,4,4,1	a	0.27	a	0.14-0.40	a	0.04	a	0.03	a	1.42	a	0.03	0.03	0.50	a
C17:1	83	83	None 2,4,4,1	None 2,4,4,1	0.27	0.27	0.12-0.42	0.12-0.42	0.05	0.05	0.03	0.03	1.88	1.56	0.03	0.03	0.72	0.59
C18:0	81	81	Standard MSC 2,4,4,1	Standard MSC 2,4,4,1	8.53	8.43	4.61-12.46	4.65-12.21	1.31	1.26	0.29	0.45	4.59	2.80	0.37	0.44	0.95	0.87
C18:1	83	83	Detrend only 2,4,4,1	SNV only 2,4,4,1	53.89	53.89	47.80-59.99	48.00-59.78	2.03	1.96	0.49	0.73	4.16	2.68	0.69	0.79	0.94	0.86
C18:1 n7	86	83	None 2,4,4,1	Standard MSC 2,4,4,1	1.85	1.86	0.84-2.86	0.91-2.82	0.34	0.32	0.24	0.21	1.42	1.50	0.32	0.22	0.50	0.56
C18:2	82	85	SNV only 2,4,4,1	None 1,4,4,1	8.86	8.92	6.27-11.46	6.05-11.79	0.86	0.96	0.19	0.33	4.65	2.90	0.37	0.34	0.95	0.88
C18:3 n6	84	82	None 2,4,4,1	Detrendonly 0,0,1,1	0.16	0.16	0.00-0.35	0.00-0.36	0.07	0.07	0.02	0.03	3.38	2.25	0.02	0.03	0.91	0.80
C18:3 n3	82	84	Standard MSC 2,4,4,1	None 2,4,4,1	0.71	0.71	0.33-1.08	0.32-1.10	0.13	0.13	0.03	0.06	4.17	2.06	0.06	0.07	0.94	0.76
C20:1	85	a	Standard MSC 2,4,4,1	a	1.54	a	1.06-2.03	a	0.16	a	0.06	a	2.66	a	0.16	a	0.86	a
C20:5 n3	79	80	None 2,4,4,1	Detrendonly 2,10,10,1	0.16	0.16	0.02-0.31	0.02-0.31	0.05	0.05	0.01	0.02	4.52	2.17	0.02	0.02	0.95	0.79
C21:0	84	82	Standard MSC 2,4,4,1	Detrendonly 2,4,4,1	0.12	0.11	0.01-0.22	0.01-0.22	0.04	0.04	0.01	0.01	2.59	2.57	0.02	0.02	0.85	0.85
C22:1	85	a	SNV only 2,4,4,1	a	0.62	a	0.42-0.83	a	0.07	a	0.04	a	1.99	a	0.04	a	0.75	a
C22:2 n6	80	76	None 2,4,4,1	None 1,4,4,1	0.08	0.08	0.00-0.36	0.00-0.36	0.09	0.09	0.03	0.03	3.53	3.03	0.03	0.04	0.92	0.89
C22:5 n3	85	80	None 2,4,4,1	Detrendonly 2,10,10,1	0.11	0.10	0.00-0.24	0.00-0.24	0.05	0.05	0.01	0.02	4.53	2.17	0.01	0.02	0.93	0.85
SFA	83	81	None 2,4,4,1	Standard MSC 2,4,4,1	29.24	29.12	20.93-37.55	20.93-37.31	2.77	2.73	0.48	0.54	5.81	5.10	1.33	0.54	0.97	0.96
MUFA	83	83	None 2,4,4,1	SNV only 2,8,6,1	60.33	60.43	54.62-66.04	54.81-66.06	1.90	1.88	0.51	0.87	3.74	2.14	0.6	0.85	0.93	0.78
PUFA	83	85	None 2,4,4,1	None 2,8,6,1	10.35	10.35	7.46-13.24	7.30-13.41	0.96	1.02	0.20	0.31	4.73	3.25	0.43	0.30	0.96	0.91
w3	82	82	None 2,4,4,1	SNV only 2,8,6,1	1.06	1.07	0.55-1.58	0.52-1.61	0.17	0.18	0.04	0.09	3.96	2.03	0.05	0.10	0.96	0.76
w6	83	84	SNV only 2,4,4,1	Detrendonly 1,4,4,1	9.37	9.35	6.60-12.13	6.57-12.13	0.92	0.93	0.19	0.32	4.80	2.89	0.28	0.32	0.96	0.88

Est. Min: estimated minimun. Est. Max: Estimated maximun. SD: Standard Deviation. RMSEC: Standard Error of Calibration. RMSEP: Standard Error of Prediciton. RSQ: multiple correlation coefficient. RPD: Ratio performance deviation: SD_{REF}/SEC.

^{a} Notdetermined

increased a significant rise in the absolute value of $\delta^{13}C$ (‰) can be observed. This is because feeding with acorns and grass conditions the $^{13}C/^{12}C$ isotope relation on corresponding with foods with C3 plants. On the other hand, as the time on the *montanera* is increased the oleic and linoleic acid contents rise while those of stearic and linolenic acid fall [4]; the relationships which include them are therefore also altered. Another source of variation is the fact of including animals with different genetics (50% and 100% Iberian) as back-fat from purebred Iberian pigs had significantly higher percentages of monounsaturated fatty acids (MUFA) and significantly smaller percentages of poly-unsaturated fatty acids (PUFA) than those from crossbred pigs [41].

3.2. Calibration equationsof $\delta^{13}C$ (‰)and fatty acids

Table 2 shows jointly the calibration descriptors of $\delta^{13}C$ (‰) and fatty acidswhen the benchtop NIR equipment and the two forms of register are used, intact subcutaneous fat (using the spectra obtained with the remote reflectance fiber-optic probe) and in the extracted fat samples (using the spectra obtained with the cam-locks), showing as N the number of samples which have been used in the model developed after the elimination of samples owing to spectral criteria (H) or chemical criteria (T), and the results of the descriptors applying the best mathematical treatment. The NIR equations allow the determination of $\delta^{13}C$ (‰) and 16 fatty acids, Σ saturated, Σmonounsaturated, Σ poly-unsaturated fatty acids, w3 and w6 in samples of extracted fat, palmitic acid, C16:0; palmitoleic acid, C16:1; heptadecanoic acid, C17:0; cis-10 heptadecen, C17:1; stearic acid, C18:0; oleic acid, C18:1; tran-vaccenic acid, 18:1 n7; linoleic acid, C18:2; γ-linolenic acid, C18:3 n6; α-linolenic acid, C18:3 n3; eicosenoic acid, C20:1; eicosapentaenoic acid, C20:5 n3; heneicosanoic acid, C21:0; erucic acid C22:1; docosa-dienoic acid C22:2 n6; DPA C22:5 n3; Σ of saturated, Σ of mono-unsaturated and Σ of polyunsaturated fatty acids together with w3 and w6. In contrast, in the case of intact samples $\delta^{13}C$ (‰) and 13 of the 16 previous fatty acids are determined as good calibration values for the minority acids C17:0; C20:1; and C22:1, together with all the summa-tions of fatty acids, were not achieved. If we observe the data in Table 2 it can be seen that the NIR calibration descriptors are good in both the samples of subcutaneous fat registered intact with fiber-optic probe and in those registered in the form of extracted fat with the cam-lock cells. The mathematical models developed for the determination of $\delta^{13}C$ (‰) and of the different fatty acids can be highlighted; they show similar margins of application and standard deviations for both forms of pre-sentation and are comparable with the results obtained by isotope ratio mass spectrometry (IRMS) for $\delta^{13}C$ (‰) and by gas chromatography for

fatty acids (Table 2). When intact samples are used the RMSEC values are slightly higher and the RMSEP values slightly lower than those of the samples of extracted fat. The RSQ values of minority fatty acids in intact samples are lower than those of the samples extracted, but always with useful values for applying the models to unknown samples. The benchtop NIR equipment showed a good prediction capacity (RPD>2) for both intact and extracted fat, excepting for some minority fatty acids such as C16:1, C17:1, C22:1. The RPD values for $\delta^{13}C$ and fatty acids were always higher when the prediction was performed on extracted fat with benchtop NIR equipment (Tables 2 and 3).

Particularly novel is the determination by NIR of the$^{13}C/^{12}C$ isotope relationship expressed as $\delta^{13}C$ (‰) in both samples of extracted fat and intact samples of subcutaneous fat, given the importance of this parameter and the cost to potential users. On the other hand, although in the bibliography it is possible to find determination by means of NIR spectroscopy of the majority fatty acids C16:0; C18:0; C18:1; C18:2, and C20:1, Σ SFA, Σ MUFA, and Σ PUFA no references have been found for the remainder of the fatty acids quantified in this study [8,32,42].

When the benchtop NIR equipment is used, MicroNIR Pro v2.5 applied to the samples of intact subcutaneous fat, the calibration de-scriptors given in Table 3 are obtained. In this table it can be observed that it is possible to quantify parameters such as $\delta^{13}C$ (‰) and 12 fatty acids such as palmitic C16:0; stearic C18:0; oleic C18:1; and linoleic acids C18:2, and other fatty acids such as C17:1, tran-vaccenic acid, 18:1 n7, α-linolenic and γ-linolenic acid, eicosenoic acid C20:1, heneicosa-noic acid C20:0, docosadienoic acid C22:2 n6, DPA C22:5 n3, Σ satu-rated, Σ monounsaturated and Σ polyunsaturated fatty acids together with Σw3 and Σ w6. Fatty acids such as palmitic, stearic, oleic, and linoleic acid were determined by using other portable NIR equipment as has been indicated in the introduction, but neither the remainder of the acids nor the summations of the various types of fatty acids [31,32]. The NIR regression models are justified by using the correlation between the concentration of each quantified parameter and the measuring at different wavelengths in accordance with the equation $y = \beta_0 + \beta_1 X_{\lambda 1} + \beta_2 X_{\lambda 2} + \beta_3 X_{\lambda 3} + \ldots\ldots + \beta_n X_{\lambda n}$, in which β refers to the coefficients and $X_{\lambda 1}$, $X_{\lambda 2}$, $X_{\lambda 3,}\ldots X_{\lambda n}$, to the wavelengths where there is a correlation. La información espectral de los ácidos grasos viene definida por una serie de bandas de absorción que de acuerdo con la bibliografía [33,42–45] corresponden a the C–H bond, which is a fundamental constituent of fatty acid molecules, absorbs strongly at wavelengths close to 1200, 1400, 1750, 2310 and 2340 nm. Se presentan ligeras diferencias en nuestro trabajo, dependiendo de la forma de registro y el equipo, pues generalmente derivan de combinaciones C–H con otros modos vibra-cionales [46]. Así, la banda observada a 1724 nm se relaciona con el

Table 3

NIR calibration statistics of $\delta^{13}C$ and fatty acids in subcutaneous fat of Iberian pigs by means of portable NIR equipment.

Constituent	N	Treatment	Mean	Est. Min	Est. Max	SD	RMSEC	RPD	RMSEP	RSQ
δ13C	81	Detrend only 2,4,4,1	−26.23	−24.93	−27.54	0.44	0.24	1.83	0.24	0.69
C16:0	80	Dentrend only 2,4,4,1	18.83	14.31	23.35	2.00	0.51	3.92	0.48	0.88
C16:1	83	Standard MSC 2,4,4,1	2.16	1.33	2.98	0.27	0.18	1.50	0.17	0.55
C17:1	78	SNV only 2,4,4,1	0.27	0.13	0.41	0.05	0.03	1.67	0.03	0.52
C18:0	78	SNV only 2,8,6,1	8.46	4.39	12.54	1.00	0.41	2.44	0.39	0.91
C18:1	81	SNV only 2,4,4,1	53.91	47.96	59.86	2.00	0.85	2.35	0.80	0.82
C18:2	81	Dentrend only 2,4,4,1	8.96	6.10	11.82	0.95	0.30	3.17	0.29	0.90
C18:3 n3	79	Dentrend only 2,4,4,1	0.71	0.31	11.14	0.13	0.06	2.17	0.06	0.79
C18:3 n6	80	None 2,4,4,1	0.16	0.00	0.35	0.06	0.03	2.00	0.03	0.77
C20:1	76	Dentrend only 2,4,4,1	0.16	0.02	0.31	0.05	0.02	2.50	0.02	0.78
C21:0	80	Standard MSC 2,4,4,1	0.11	0.007	0.22	0.04	0.02	2.00	0.02	0.75
C22:2 n6	72	SNV only 2,4,4,1	0.08	0.00	0.37	0.10	0.06	1.67	0.06	0.86
C22:5 n3	80	SNV only 2,4,4,1	0.10	0.00	0.24	0.05	0.02	2.50	0.02	0.77
SFA	80	Dentrend only 2,4,4,1	29.22	20.85	37.58	3.00	0.68	4.41	0.64	0.94
MUFA	81	None 1,4,4,1	60.38	54.55	66.21	2.00	0.80	2.50	0.75	0.83
PUFA	77	Standard MSC 2,4,4,1	10.40	7.38	13.41	1.00	0.30	3.33	0.28	0.91
w3	77	None 2,4,4,1	1.06	0.52	1.61	0.18	0.08	2.25	0.07	0.80
w6	81	None 2,4,4,1	9.36	6.58	12.13	0.94	0.30	3.13	0.28	0.89

Est. Min: estimated minimum. Est. Max: Estimated maximun. SD: Standard Deviation. RMSEC: Standard Error of Calibration. RMSEP: Standard Error of Prediciton. RSQ: multiple correlation coefficient. RPD: Ratio performance deviation: SD_{REf}/SEC.

enlace C=O en los lípidos, a esa longitud de onda, para el ácido oleico en grasa extraida el valor de β es muy elevado, 6519.9, que justificaría la mejor calibración de este parámetro en grasa extraida, en comparación con la obtenida en muestras intactas. Resultados similares a los encontrados por otros autores [33,47], que observan que la banda 1720–1730 aumenta cuando lo hace la insaturación en los ácidos grasos; además de producirse un desplazamiento a longitudes de onda mas larga cuando aumenta la longitud de la cadena. A 2380 nm, zona del espectro NIR que corresponde a las bandas de combinación C–H de los ácidos grasos [42, 44], existe correlación, entre otros, para el ácido alfa linolenic, con un valor de β de 472.4 en muestras de grasa extraida, que justifica la calibración en este tipo de muestras.

Si hacemos referencia a los los modelos de calibración obtenidos para $\delta^{13}C$ nos encontramos que en la zona del espectro registrada únicamente con el equipo portatil (de 908 nm a 1100 nm) a 1000 nm presenta valores β de 168, correspondientes al second overtone de NH_2, –NH de secondary amines y a 1069 nm, con valores de β, de 153,5, correspondientes a –$CONH_2$ primary amides [48]; que podrían estar relacionados con la alimentación de los cerdos en montanera (bellota, hierba)?? Cuando buscamos correlaciones de $\delta^{13}C$ con la sonda de fibra óptica, de nuevo se encuentra correlaciones fuertes a 1356 nm y valores de β de 2218, relacionados con combinación de grupos –CH_3 methyl, a 1532 nm con β de 1161 (correspondientes a enlaces N–H stretch y first overtone de enlaces R–NH_2 [44]) y a 1820 nm con valores de β de 1281. Todas estas correlaciones justifican la determinación del parámetro $\delta^{13}C$ en muestras de grasa subcutánea.

Tambien resulta interesante comprobar que en la zona del espectro obtenido con el equipo portatil entre 1010–1100, se obtienen coeficientes β para los Σmonounsaturated, Σ saturated, Σ polyunsaturated fatty acids de 574, 537 y 143, correspondientes a los grupos –CH_3 combination y CH_2 combination [48].

This will justify the fact that the NIR spectra obtained with extracted fat where the area of the spectrum is wider (1100–2498) show improved correlations with the parameters under study than those obtained with intact subcutaneous fat with a fiber-optic probe register, the spectrum area of which is of 1100–2000 nm. Furthermore, the results obtained with the benchtop equipment, the RSQ values, are slightly lower than those achieved with other forms of register.

3.3. Internal and external validation

The internal assessment of NIR calibration models is estimated by cross-validation. In this method the set of calibration values is divided into a series of subsets. The number of cross-validation groups found by using the benchtop NIR equipment register was 8 in the intact samples of subcutaneous fat and 7 for the extracted fat samples, and for the samples registered with the portable equipment 8 in the case of the intact subcutaneous fat samples. From these a subgroup is taken for prediction and the remainder for calibration, repeating the process so that all the subgroups have passed through prediction and calibration. For all the constituents calibrated by means of MPLS with the benchtop NIR equipment (Table 2) and portable NIR equipment (Table 3), the standard errors of prediction (RMSEP) can be seen in Tables 2 and 3 together with the calibration parameters, the margin of application, the RSQ, and the standard error of calibration (RMSEC). It can be observed that the prediction errors are of the same type as those of calibration (RMSEC), which is an indicator that the NIR models obtained are suitable. The comparison of the NIR data obtained by means of the mathematical models and the reference data for $\delta^{13}C$ (‰) and some fatty acids are presented in Fig. 2. We provide as a comparison the internal validation graphs for the parameters $\delta^{13}C$ (‰), oleic acid C18:1, linoleic acid C18:2, and α-linolenic acid, both intact subcutaneous fat in the two

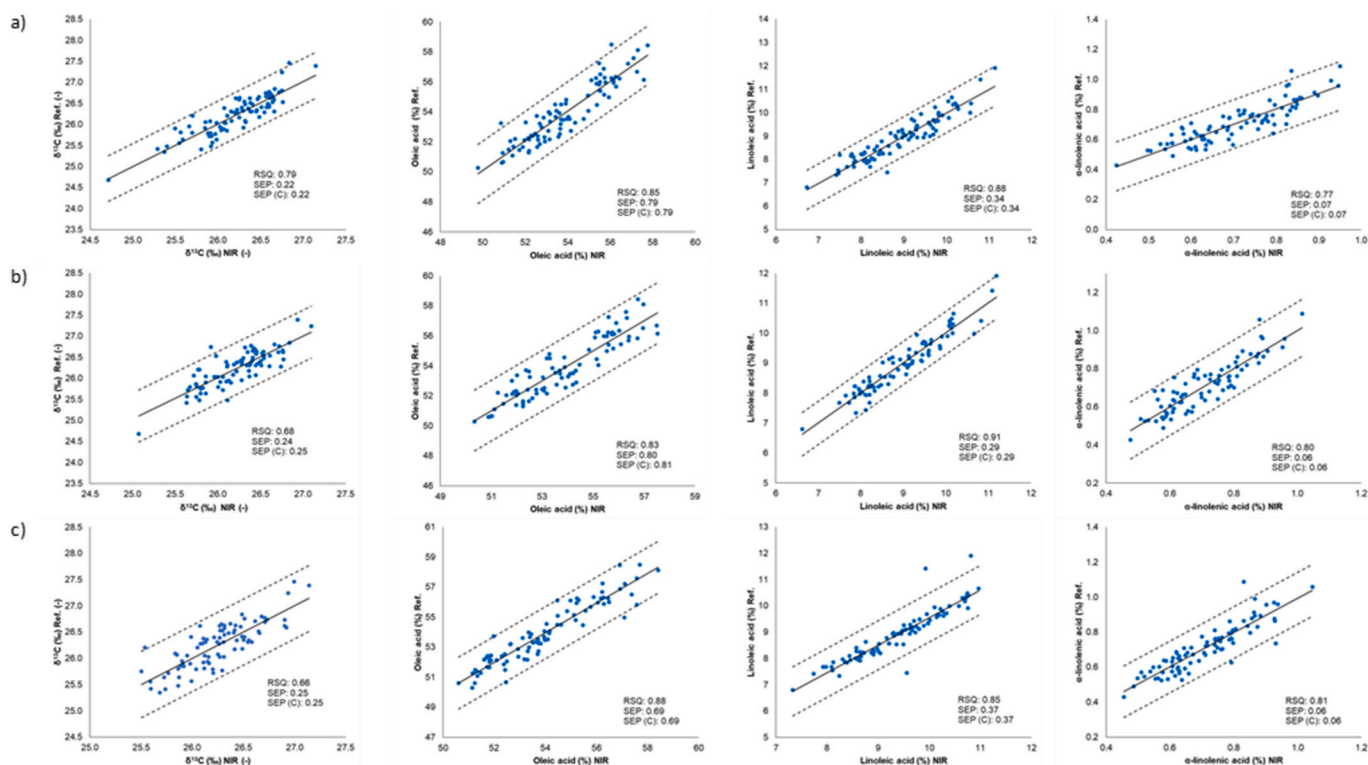**Fig. 2.** Internal validation. Comparative study of $\delta^{13}C$ and Oleic acid a) Foos NIR System 5000 with a fibre-optic probe. Samples intact subcutaneous fat b) MicroNIR Pro v2.5. Samples intact subcutaneous fat and c) Foss NIR System 5000 with a "Cam-lock" probe. Samples of extracted subcutaneous fat.
Fig. 2 (continuation). Internal validation. Comparative study of linoleic and α-Linolenic acid. a) Foos NIR System 5000 with a fibre-optic probe. Samples intact subcutaneous fat b) MicroNIR Pro v2.5. Samples intact subcutaneous fat and c) Foss NIR System 5000 with a "Cam-lock" probe. Samples of extracted subcutaneous fat.

forms of register (benchtop equipment with fiber-optic probe and portable equipment) and extracted subcutaneous fat registered with the cam-lock cells with the benchtop NIR equipment. From this it can be inferred that the NIRS technique is a useful alternative that is comparable to the spectrometry of isotope masses and gas chromatography for the determination of the content of the parameters here established in samples of subcutaneous fat with both benchtop and portable NIR equipment.

In external validation the solidity of the method is checked by applying NIRS technology to 20 new samples of subcutaneous fat which were not used in the calibration models. The procedure was as follows: the spectra were registered in triplicate and the spectral average was taken, the equations obtained were applied, and the aforementioned values were compared with the reference in accordance with the residuals and the Root Mean Square Error (RMSE). Table 4 shows the results obtained in the external validation (20 samples), with the Root Mean Standard Error (RMSE) and RMSEP values, for the three forms of measurement. Moreover, with the analysis of the Student t-test for matched values, the values of δ^{13}C (‰) and fatty acids obtained with the mass spectrometer and chromatography were compared with those mentioned above in external validation with the NIRS model (with both the benchtop and portable NIR equipment). These values were higher than the minimum significance level of 0.05. The null hypothesis is therefore accepted and it can be said that there is no difference between the values of δ^{13}C (‰) and fatty acids generated by the different methods.

3.4. Comparison of results for benchtop and portable NIR equipment

When the same samples of subcutaneous fat are used in the register of both portable and benchtop NIRS equipment, the potential of the portable equipment MicroNIR Pro v2.5 is assessed. When the calibration models are developed as has been mentioned, by using the portable equipment it is possible to determine δ^{13}C (‰) and 12 fatty acids in addition to the 5 summations according to their functional group (Σ saturated, Σmonounsaturated, Σ polyunsaturated fatty acids, Σw3, and Σw6). When the benchtop equipment is used one can determine, both with intact subcutaneous fat samples and extracted fat samples, δ^{13}C (‰) and 16 acids and the 5 summations of interest (Σ saturated, Σmonounsaturated, Σ polyunsaturated fatty acids, Σw3, and Σw6). It can be pointed out that the C17:0 and C20:1 acids can only be determined for the extracted fat in the benchtop NIR equipment, whereas the C20:1 acid can be determined with the portable equipment although the quantification margins are narrower. The mean values and the margins (minimum estimate-maximum estimate) within which it is possible to quantify the various constituents in the different forms of register used are similar and analogous to the chemical data of spectrometry of isotope masses for the values of δ^{13}C (‰) and the fatty acids obtained by gas chromatography. The RSQ values are always higher when benchtop NIR equipment and extracted fat is used compared with those obtained with the register of remote reflectance fiber-optic probe, which had been found in previous studies [7]. When portable NIR equipment is used the RSQ values for the constituents when compared with the same type of sample, intact subcutaneous fat, are slightly lower except in the case of C18:0 and C18:3 n6, which are slightly higher. The RPD and prediction errors are along the same lines.

4. Conclusion

Based on the results obtained in this study we confirm the suitability of the portable NIR equipment, MicroNIR Pro v2.5, for the determination in intact subcutaneous fat of Iberian pigs at the time of slaughter of δ^{13}C (‰) and of some fatty acids such as palmitic, C16:0; stearic C18:0; oleic C18:1; and linoleic C18:2, α-linolenic and γ-linolenic acids but also other fatty acids such as eicosenoic acid C20:1, heneicosanoic acid C20:0, docosadienoic acid C22:2 n6, DPA C22:5 n3 together with Σ of

Table 4
The external validation (20 samples), with the Root Mean Standard Error (RMSE) values and RMSEP
Samples of Intact Subcutaneous Fat (ISF); Extracted subcutaneous fat (ESF) and Portable NIR.

Constituent	RMSE			RMSEP		
	ESF	ISF	Portable NIR	ESF	ISF	Portable NIR
δ13C	0.03	0.01	0.02	0.38	0.27	0.32
C16:0	0.56	0.23	0.68	0.95	0.46	0.80
C16:1	0.01	0.01	0.02	0.21	0.24	0.27
C17:0	0.00	*		0.05	*	
C17:1	0.00	0.00	0.00	0.04	0.04	0.04
C18:0	0.47	0.29	0.50	0.82	0.65	0.88
C18:1	2.93	0.91	1.25	1.84	0.86	1.02
C18:1 n7	0.03	0.02		0.45	0.29	
C18:2	0.40	0.10	0.17	0.82	0.41	0.47
C18:3 n6	0.00	0.00	0.00	0.06	0.05	0.04
C18:3 n3	0.00	0.00	0.00	0.10	0.08	0.09
C20:1	0.01	*	0.00	0.26	*	0.05
C20:5 n3	0.00	0.00		0.03	0.03	
C21:0	0.00	0.00	0.00	0.03	0.02	0.03
C22:1	0.00	*		0.08	*	
C22:2 n6	0.00	0.00	0.00	0.08	0.05	0.08
C22:5 n3	0.00	0.00	0.00	0.04	0.03	0.03
SFA	4.28	0.83	4.03	1.76	0.74	1.48
MUFA	3.37	0.06	2.23	1.76	1.13	1.57
PUFA	0.52	0.05	0.20	0.93	0.44	0.53
w3	0.00	0.00	0.00	0.11	0.11	0.13
w6	0.36	0.10	0.15	0.77	0.45	0.44

saturated, Σ of monounsaturated and Σ of polyunsaturated fatty acids both Σw3 and Σw6. The measurement of δ^{13}C (‰) is related to the time of the *montanera* (acorns and grass) received by the Iberian pig as well as the levels of the fatty acids. The results are comparable to those obtained for the same intact subcutaneous fat sample with benchtop NIR equipment, Foss NIR System 5000, with remote reflectance fiber-optic probe. It should be noted that prediction capacity for δ^{13}C and fatty acids for both intact subcutaneous fat and extracted subcutaneous fat was always higher when these parameters were determined with the becnchtop NIR equipment. This indicates that portable equipment may be an alternative to benchtop equipment, which is fixed, large, and more expensive, for the quantitative control of the parameters of interest of the Iberian pig.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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